Research Article

In Vivo Osteogenesis of Vancomycin Loaded Nanohydroxyapatite/Collagen/Calcium Sulfate Composite for Treating Infectious Bone Defect Induced by Chronic Osteomyelitis

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A novel antibacterial bone graft substitute was developed to repair bone defects and to inhibit related infections simultaneously. This bone composite was prepared by introducing vancomycin (VCM) to nanohydroxyapatite/collagen/calcium sulphate hemihydrate (nHAC/CSH). XRD, SEM, and CCK-8 tests were used to characterize the structure and morphology and to investigate the adhesion and proliferation of murine osteoblastic MG63-E1 cell on VCM/nHAC/CSH composite. The effectiveness in restoring infectious bone defects was evaluated in vivo using a rabbit model of chronic osteomyelitis. Our in vivo results implied that the VCM/nHAC/CSH composite performed well both in antibacterial ability and in bone regeneration. This novel bone graft substitute should be very promising for the treatment of bone defect-related infection in orthopedic surgeries.

1. Introduction

Bone defect-related infections especially chronic osteomyelitis are quite common in open fracture and trauma in clinical treatment, which continues to be very difficult to treat and brings challenges to clinicians. It is difficult to make effective bone repair and inhibit infection at the same time. Vancomycin hydrochloride (VCM) is an antibiotic drug that is specifically used against Staphylococcus aureus in the treatment of bone-related infections. However, systemically administered antibiotics have been associated with a number of difficulties including toxic side effects if the drug level is too high or may fail to exert the proper therapeutic effect if the drug level is too low at the site of need. These disadvantages could be markedly reduced if the antibiotic is applied locally at the site of infection by incorporating it into, or onto, implantable skeletal delivery scaffold, and it may also improve efficacy by delivering valid and safe drug concentrations to the infected bone.

Therefore, it is critical to select an appropriate scaffold for constructing drug delivery system, in which the scaffold should be biocompatible, osteogenic, operable, biodegradable, and antibacterial. Primarily, the scaffold should possess suitable ingredients and structure for cell attachment, proliferation, and osteogenic differentiation. Li et al. did some related studies [1–3].

PMMA is often incorporated with antibiotics such as gentamicin as one of the most widely used bone materials in clinical applications. However, the inherent biological inertia of PMMA leads to poor osseointegration between bone tissue and cement interface, apart from other shortcomings such as nonabsorbability, impermeability to antibiotic, monomer
toxicity, and high polymerization temperature [4–8]. Because of these drawbacks, several absorbable, osteoconductive, and low reaction temperature antibiotic carriers have been developed for inhibiting infection as well as avoiding a second surgery [9–14].

Calcium sulfate hemihydrate (CSH) itself has a long clinical history as a bone graft substitute, known for its bioreabsorption, satisfactory handling properties, or self-setting ability in situ after filling the defect. After mixing, CSH form a viscous moldable paste, which in some instances can be injected during surgery using minimally invasive procedures. Moreover, the setting reaction of CSH is not obviously exothermic as PMMA, which are widely used in orthopedic surgery, especially for arthroplasty fixation and vertebroplasty. Therefore, the incorporation of different drugs and biological molecules makes them good candidates for drug delivery applications in bone tissue engineering [15]. However, there are also some drawbacks with the calcium sulfate material including its insufficient ability to stimulate bone regeneration. CSH cement cannot form a chemical bond with bone tissue at the early stage of therapy because of its poor bioactivity [16–19]. Ideal antibiotic carriers should be able to promote early mineralization and support new bone formation and simultaneously control the release of drug.

To optimize the performance of bone regeneration of CSH, some previous studies in our group suggested that nanohydroxyapatite/collagen (nHAC) could be incorporated, which was prepared from mineralizing type I collagen with excellent osteoconductive properties, and was thought to be a new scaffold material for its high similarity of natural bone both in composition and in hierarchical nanostructure in bone tissue engineering [20–25]. It has been demonstrated that nanostructured materials, compared with conventional materials, may promote greater amounts of specific protein interactions, thereby more efficiently stimulating new bone formation. It has also been indicated that when features or ingredients of scaffolds are nanoscale, a variety of interactions can be stimulated at the cellular level [26–29].

The aim of the present study was to investigate nHAC/CSH as a carrier material for vancomycin in the treatment of chronic osteomyelitis to repair bone defects and inhibit related infections simultaneously. An in vivo study of the efficacy of this drug-loaded bone cement was performed in an experimental model of chronic bone infection caused by MRSA.

2. Materials and Methods

2.1. Material Preparation and Physicochemical Properties Characterization. The powders of nHAC and CSH were prepared as previously described. In brief, nHAC was obtained by precipitation of Ca$^{2+}$ and PO$_4^{3−}$ on the collagen molecule from its precursors (CaCl$_2$ and Na$_2$HPO$_4$), followed by product freeze-drying. And the CSH were prepared from CSD (calcium sulphate dehydrate) by hydrothermal synthesis [30, 31]. In this study, the VCM/nHAC/CSH were prepared by mixing 5 wt% VCM-HCl, 5 wt% nHAC, and 90 wt% CSH uniformly. Control samples (nHAC/CSH) were also prepared using the same process without VCM. The samples were mixed with deionized water at 0.5 ratio of liquid to solid (L/S) and stirred to form homogeneous paste within 20 s and then stored in 100% humidity at 37°C to set.

The composition of materials was characterized by X-ray diffraction (XRD, D/Max-2500X) using monochromated CuKα radiation (λ = 1.5405 Å, 120 mA, 40 kV) in a continuous scan mode with a scanning speed of 8°/min, and the 2θ range was from 10° to 90°.

Composite samples were sputter-coated with gold film for scanning electron microscopy (SEM, Quanta 200 FEG, Netherlands) examinations at the voltage of 20 kV.

2.2. Cell Experiment. In order to evaluate the biocompatibility of cement, cell counting kit-8 (CCK-8) method was used to quantitatively evaluate the proliferation of murine osteoblastic MC3T3-E1 (a clonal osteogenic cell line derived from newborn mouse calvarias, which is often used in bone tissue engineering research). Cell growth and adhesion behaviors on the scaffolds surfaces were examined by SEM observation. MC3T3-E1 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air incubator. Two types of composite cements (nHAC/CSH and VCM/nHAC/CSH) were cut into small cubes (with 5 mm in diameter and 2 mm in thickness) for 96-well plate, sterilized by $^{60}$Co γ-irradiation at the dose of 16 kGy. The cells were seeded onto the sterilized cements at a density of 1 × 10$^5$ cells/mL, and 100 μL of such cell suspension was added to each well. For SEM examination (JSM-T300, JEOL, Tokyo, Japan), cells were collected after 24 h incubation and then fixed with 2.5% glutaraldehyde in PBS for 0.5 h. After dehydration with a series of graded ethanol (30, 50, 70, 80, 90, 95, and 100%), the samples were critical-point-dried and then coated with gold film for SEM examination. In order to evaluate cell proliferation, CCK-8 assay (Dojindo Molecular Technologies Inc.) was performed according to the manufacturer’s protocol. Briefly, after incubating at 37°C and 5% CO$_2$ for indicated time (1, 3, and 5 days), the culture medium was replaced with 100 μL fresh medium containing 10 μL CCK-8 solution in each well for another 3 h incubation before measurement at 450 nm with a micro plate reader (Bio-Rad, Model 680). Four repeated measurements for each time point of each group (n = 4) were carried out for statistical analysis.

2.3. In Vivo Study. The animal experiments were carried on by the approval of the Ethics Committee of the General Hospital of People’s Liberation Army. The in vivo study was conducted on New Zealand rabbits (3.5–4.0 kg). Bone infection was induced in the condylus lateralis femoris in 30 rabbits using the model of Norden et al. under general anaesthesia. The site of operation was shaved and cleaned with alcohol. The medullary cavity was exposed through a lateral approach and a small hole was made (Φ7 mm × 9 mm). 0.2 mL 5% sodium morrhuate was directly delivered into the cavity followed by 0.2 mL bacterial suspension with...
1 × 10⁸ CFU/mL S. aureus (MRSA, ATCC 43300, Shanghai Harmony Biotechnology Co., Ltd.). The wound was closed by suturing. Three weeks after induction of infection, all rabbits were infected and treated by focal debridement. Control group were injected by nHAC/CSH loading without antibiotics, whereas animals of treatment group were injected by VCM/nHAC/CSH for 3 months.

After surgery, rabbit femoris in defect regions were extracted and fixed in 4% paraformaldehyde and then decalcified in EDTA and dehydrated in ethanol before they were embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin and Masson’s trichrome. Histomorphometry was carried out using a light microscope (BX51, Olympus) under 40x magnification. For the evaluation of fibrosis, picrosirius red staining was performed using 0.1% picrosirius red solution. Micro-computed tomography was used to observe the bone reconstitution.

2.4. Statistical Analysis. All the data were statistically analyzed using SPSS 13.0 software and expressed as the standard deviation of the mean. The t-test was performed and p < 0.05 was commonly accepted to be statistically significant.

3. Result and Discussion

3.1. Characterization of the Physicochemical Properties of VCM/nHAC/CSH Scaffold. The conversion of CSD phase to α-CSH phase by hydrothermal synthesis was confirmed by XRD analyses (Figures 1(d)(A) and 1(d)(B)) and revealed complete transformation of CSD with new diffraction peaks located at 14.75°, 25.58°, 29.76°, and 31.94°, which were correlated with the characteristic crystal planes of 110, 310, 220, and −114 for α-CSH phase.

Also the characteristic peaks of nHAC located at 25.91°, 31.82°, 32.20°, 32.93°, and 34.10° and correlated with crystal planes of 002, 211, 112, 300, and 202 (Figure 1(d)(C)) were shown. The broadening of the diffraction peaks of nHAC implied the small grain size and low crystallinity. nHAC powder was composed of some irregular particles as shown in Figure 1(b). The similarities to natural bone in the microstructure of nHAC have been verified via conventional and high-resolution transmission electron microscopy by Zhang et al. [32]. After the hydration reaction from VCM/nHAC/CSH powder to set cement, the XRD peaks of which were changed to 11.64°, 20.75°, 23.41°, and 29.14°, corresponding to crystal planes of 020, 021, 040, and 041. Such changes indicated that the CSH with rod-like structure
in the powder was transformed into CSD with sheet crystal structure in the set cement during the hydration reaction, as shown in Figures 1(b) and 1(c).

In our previous study, it was demonstrated that final setting time was about 15∼20 min. The porosity of the scaffold was 38.8% and the compressive mechanical strength was about 4.8 MPa, which was more than the lower limit of natural cancellous bone (1 MPa) [31].

3.2. CCK-8 Assay. In order to evaluate the cell toxicity of VCM, CCK-8 method was used to measure the proliferation of MC3T3-E1 cells on the scaffold surfaces. Figure 2(a) shows the proliferation results of cells cultured for 1, 3, and 5 days, respectively. For all times, the OD values of VCM/nHAC/CSH were not significantly lower than that of the nHAC/CSH for each point in time. This is not surprising since the antibacterial property of VCM is also expected to have an adverse effect on the osteoblastic cell viability. However, it was also shown that the cell growth on both groups increased significantly from 1 day to 5 days (p < 0.05). Beyond that, cells performed well in attachment and spreading on both of bone materials with multiple filopodia. These results indicated that the VCM/nHAC/CSH scaffold with 5 wt% VCM addition had satisfied in vitro biocompatibility.

3.3. In Vivo Study. In the previous study, the materials were shown to be in vitro antibacterial. The inhibition ratio of VCM/nHAC/CSH was more than 99.8% and the distinct inhibition zone of 18 mm was formed in Staphylococcus aureus bacterium incubation dish with VCM/nHAC/CSH disc in the center of agar matrix for 16 hours of incubation. On HE and Masson histological analysis at 12 weeks after implantation, single islands of new trabecular bone formed, and a number of active nonaligned osteocytes and osteoblasts at the periphery of bone trabecula were frequently observed in the VCM/nHAC/CSH group. No signs of necrosis and inflammation were found in the group of VCM/nHAC/CSH at this time. By contrast, all rabbits revealed evidence of chronic infections, and there were many bone necroses in the control group. Histological observation in this study demonstrated the effectiveness of treatment in an experimental
model of chronic bone infection caused by MRSA within 12 weeks.

Picrosirius red staining is one of the best techniques of collagen histochemistry. Sirius red enhances the birefringence in oriented collagens as it attaches to collagens in parallel; then we can see the red-orange colored light from sirius red stained collagens in polarized light microscopy. As the enhancement of birefringence is limited in fibrous tissues, this method can be used to identify fibrous collagen. In this study, the tissues were fixed in a solution of 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with picrosirius red. There were more fibrous tissues in the VCM/nHAC/CSH group than that in nHAC/CSH group in picrosirius red stained cross section after infection with MRSA following 12 weeks of treatment as shown in Figures 3(e) and 3(f).

The results also were confirmed by the micro-computed tomography graphs of implanted materials on the antibacterial bone defect as shown in Figure 4. After 12 weeks, the bone tissues were destroyed in different places of rabbit
femoris (pointed with the arrows in Figure 4(c)) because of infection without drug release in nHAC/CSH group. But, in the treatment group (Figure 4(d)), the bone reconstruction was better than control group (Figure 4(c)) and close to normal femoris bone (Figure 4(b)).

All results above suggested that the inflammation may actually inhibit the growth of bone tissue in nHAC/CSH group, and the treatment of inflammation along with bone repair was effective in VCM/nHAC/CSH group.

The best treatment of contaminated or infected bone defects, such as chronic osteomyelitis, is to control infection and repair bone defect at the same time. This requires an osteoinductive bone graft composite with ideal release antibiotic capabilities, mechanical properties, and other related properties. In this study, the nHAC/CSH was used as a carrier of vancomycin (VCM) for the treatment of osteomyelitis, and the VCM/nHAC/CSH composite has ideal self-setting, antibacterial, porous, degradable, good mechanical properties, and better osteogenic activity. So the materials can act not only as void filler facilitating tissue regeneration but also as carrier for inhibiting infection in the healing process.

The bioactivity of the scaffold was determined by the components such as CSH and nHAC, of which CSH as the main ingredient has been used in bone augmentation for many years in virtue of its self-setting ability in situ as well as filling the defect. One major drawback in the use of calcium sulphate is insufficient osteogenic activity. Calcium ions were released during dissolution of calcium sulfate. Its dissolution leads to acidic microenvironment responsible for local inflammatory processes at the site of implantation in human bone. Inflammatory tissue was found to disappear after 60 days in bone but to remain in soft tissue implantation sites of white New Zealand rabbits [33]. On the other hand, local increases in calcium ion concentration may affect osteoblast genesis and function, and they may act as a stimulus to osteoblast differentiation. Besides, in live tissue, HA and collagen are the major mineral and organic component of human bones. Recently, various hydroxyapatite (HA) or collagen based composites were developed as potential biomaterials for bone substitutes due to their compositional analogy to bone [34]. Some research uses modified HA as antimicrobial coatings or carrier [35]. In this paper, nHAC was added to calcium sulphate to enhance biocompatibility of calcium sulphate in a composite material, which was developed based on biomimetically synthetic mineralized collagen fibrils. The inorganic phase in the composite is carbonate-substituted HA with low crystallinity as well as nanometer size, and organic phase of type I collagen matrix has a characteristic quarter staggered arrangement of tropocollagen molecules assembled and aligned with an axial period of approximately 67 nm. The matrix serves as a template for orderly deposition of mineral platelets [28]. Studies have demonstrated that nanostructured materials with cell favorable surface properties may promote greater amounts of specific protein interactions to more efficiently stimulate new bone growth compared to conventional materials [29]. The composite has been proven to be with unique osteoinduction and osteoconduction and successfully applied in clinical treatment.

Since the 1980s, vancomycin has become the first choice for treating refractory hip infections because of its high efficacy against gentamicin-resistant bacteria, its rare bacterial resistance, and its low incidence of side effect [8]. In this study, vancomycin can be mixed with powdered nHAC/CSH at the site of bone defect and sustained release on the course of operation. However, loading is not limited to one specific antibiotic (e.g., tobramycin or gentamicin) but can be done according to antibiograms offering individual treatment options. Calcium sulphate's capillary porosity also can be used for impregnation of already hardened calcium sulphate pellets with various antibiotic solutions, which would be reported in our subsequent research.

In in vivo study, after three weeks of induction of infection and 12 weeks of treatment with VCM/nHAC/CSH, no evidence of infection or foreign body reaction was observed. The scaffold had almost been completely degraded and a lot of new trabecular bones had formed in the implant site. By contrast, all rabbits in the control groups of nHAC/CSH showed evidence of chronic infections.

4. Conclusion

In this study, we first used the VCM/nHAC/CSH bone substitute as a degradable local antibiotic delivery system for the treatment of chronic osteomyelitis. The implants were successful as vancomycin carriers in inhibiting infection in rabbits osteomyelitis mode during the course of this study. Moreover, the implants performed excellent biocompatibility. Our results suggested that the implants can be considered a new system for local vancomycin delivery and the effectiveness of VCM/nHAC/CSH bone substitute in the treatment of S. aureus induced chronic osteomyelitis and simultaneously stimulated bone regeneration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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