Osteoinductive and Antibacterial Biomaterials for Bone Tissue Engineering

Wang, D.

2016

Chapter 3

A novel bone-defect-filling material with sequential antibacterial and osteoinductive properties for repairing infected bone defects

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In preparation

Abstract

Objectives: The repair of infected critical-sized bone defects is hindered by both active infection of residual bacteria and compromised bone regenerative capacity. We recently develop biomimetic calcium phosphate (BioCaP) granules that can internationally incorporate and slowly release osteoinductive bone morphogenetic protein-2 (BMP2) to efficiently induce new bone formation. To provide a viable treatment option for infected bone defects, we hereby develop a novel bone-defect-filling material with a sequential release system: burst release of a powerful antibacterial agent-Hydroxypropyltrimethylammonium chloride chitosan (HACC) and followed by a controlled release of BMP2. We hypothesize that BMP2-BioCaP/HACC complex can rapidly kill residual bacteria and thereafter induce new bone formation so as to repair infected bone defects.

Materials and methods: The minimum bactericidal concentration (MBC) of HACC against infection-associated bacteria and cytotoxicity of HACC to pre-osteoblasts were both evaluated to determine the optimal concentration of HACC. The influence of HACC at the optimal concentration on the BMP2-induced differentiation of pre-osteoblasts was evaluated by assessing alkaline phosphatase (ALP) activity, osteocalcin (OCN) expression and mineral nodule formation. Corresponding amount of was then adsorbed on BMP2-BioCaP granules and air-dried to obtain BMP2-BioCaP/HACC complex. The release kinetics of HACC and BMP2 were determined using quantitative spectrophotometric analysis. To evaluate osteoinductivity of BMP2-BioCaP/HACC complex and optimize the HACC concentration in vivo, the following groups (n=6 samples, 100uL per sample) were randomly implanted into subcutaneous pockets in rats: 1) BMP2-BioCaP/HACC complex (BMP2-BioCaP/HACC complex carrying 20, 4 or 0.8μg HACC were respectively named as BMP2-BioCaP/HACC-20, BMP2-BioCaP/HACC-4, BMP2-BioCaP/HACC-0.8); 2) BMP2-BioCaP; 3) BioCaP/HACC; 4) BioCaP. Five weeks after implantation, samples were retrieved for histological and histomorphometric analysis.

Results: The optimal HACC concentration is 40μg/mL, at which HACC can kill bacteria without harming pre-osteoblasts. 40μg/mL HACC didn't significantly influence the BMP2-induced ALP, OCN and mineralization. The in-vitro release profile showed that HACC was completely exhausted after 3 days, while BMP2 was gradually and slowly released with about 20% depletion after 30 days. In the in-vivo pro-fibrotic environment (subcutaneous sites), bone formation was observed only in the BMP2-containing groups. Furthermore, in comparison with the positive control group (BMP2-BioCaP), BMP2-BioCaP/HACC-4 and BMP2-BioCaP/HACC-0.8 complex resulted in similar amount of new bone formation, while BMP2-BioCaP/HACC-20 was associated with significantly less new bone formation.

Conclusion: BMP2-BioCaP/HACC complex could rapidly eliminate antibiotic-resistant bacteria and efficiently promote new bone formation both in vitro and in vivo, which conferred this novel material a promising application potential to repair infected bone defects.

Keywords: Bone morphogenic protein-2, Hydroxypropyltrimethylammonium chloride chitosan, Antibacterial activity, Osteoinductivity, Bone repair

1. Introduction

Achieving satisfactory bone regeneration in infected bone defects is a great challenge in the field of orthopaedic, oral and maxillofacial surgery. The infected bone defects are often caused by trauma combined with direct contamination. Such acute bone infections sometimes progress to chronic infections (clinically referred to as osteomyelitis). Routine systemic antimicrobial therapy is typically sufficient to clear an acute bone infection; but chronic osteomyelitis can be extremely difficult to treat and requires radical surgical debridement of the necrotic and infected tissues, followed by extensive systemic application of antibiotics [1, 2]. This radical debridement process often results in a large bone and soft tissue defect, where the dead space must be effectively managed to gain tissue regeneration and so as to reduce the chance of reinfection [3, 4]. If the bone defect is too large and beyond the intrinsic self-healing capacity, which is considered as critical-sized bone defects, bone grafting is needed to facilitate bone regeneration. Although autografts are regarded as “gold standards” for repairing bone defects [5, 6], their application is limited because of its low availability and donor-site pain and morbidity [7]. Synthetic calcium phosphate (CaP)-based materials are therefore widely adopted to heal bone defects clinically [8, 9]. This is due to its excellent osteoconductivity [10-12]—the ability to facilitate the migration and proliferation of osteoblasts and progenitor cells [13]. However, they lack osteoinductivity—the ability to induce progenitor cells to differentiate down osteogenic lineages [13], which is essential for satisfactory bone regeneration in critical-sized bone defects. One approach to solving this problem is to introduce osteoinductive agents into CaP materials, such as bone morphogenetic protein 2 (BMP2). To maximize its osteoinductivity, BMP2 needs to be delivered to target sites at low concentration in a sustained manner [14]. Our research group has recently developed BMP2-incorporated biomimetic calcium phosphate (BMP2-BioCaP) which could slowly and continuously deliver BMP2. The BMP2-BioCaP granules were proved to be an osteoinductive bone substitute, which can induce bone formation efficiently in subcutaneous pocket model in rats [15].

Despite the aggressive tissue debridement, the spatial heterogeneity of bacterial

colonization in the bone and the surrounding tissue makes it impossible to ensure complete elimination [16]. The residual bacteria release various inflammatory mediators and tissue-destructive enzymes, which can compromise bone regeneration [17]. Therefore, to gain favorable bone reconstruction, it is necessary to locally use bactericidal or bacteriostatic antibiotics to stop bacteria from secreting inflammatory mediators. The most often used antibiotics are vancomycin, gentamicin and tobramycin. However, their application is more and more limited due to the emergence of resistance in bacteria in hospitals worldwide by use and misuse of these conventional antibiotic [18]. On the other hand, a novel water-soluble chitosan derivative (hydroxypropyltrimethyl ammonium chloride chitosan, HACC) has attracted much attention due to its strong antibacterial activity, broad spectrum [19, 20] and very few reported bacterial resistance [21], which made it a promising candidate antibiotics for treatment of infected bone defects [22].

To optimize the local delivery of antibiotics, much focus has been put on developing antibiotics-loaded biomaterial system to help eradicate an established bone infection. Poly(methyl methacrylate) (PMMA) bone cement is the gold standard biomaterial for local antibiotic therapy in orthopaedics and has been used for over 35 years for both prophylaxis and therapy. Seminal studies conducted by Buchholz and Engelbrecht in 1970 [23] and Klemm in 1979 [24] described the use of antibiotic-loaden PMMA to prevent infection and treat chronic bone infections, respectively. However, PMMA lacks degradability, which is troublesome due to unfavorable release patterns of the antibiotic as well as necessity of a second surgery to remove it and to implant bone grafts. The ideal approach to solve this problem is to develop a biodegradable local biomaterial system, functioning as both carrier for antibiotics and bone grafts, to not only clear the infection but to also contribute to the subsequent bone regeneration process [16].

In the present study, to develop a favorable local biomaterial system for the treatment of infected critical-sized bone defects, we used our previously developed osteoinductive bone substitute, BMP2-BioCaP granules, as antibiotic carrier. HACC solution was then adsorbed on them and air-dried to obtain an antibacterial and osteoinductive biomaterial system─BMP2-BioCaP/HACC complex. It can achieve sequential release of antibiotic and osteoinductive agent— a burst release of HACC and following slow and sustained release of BMP2. We hypothesized that the BMP2-BioCaP/HACC complex could rapidly eliminate residual bacteria and thereafter induce new bone formation so as to repair infected critical-sized bone defects.

2. Materials and methods

2.1 Antibacterial assay of HACC

Minimum bactericidal concentration (MBC) against methicillin-resistant *staphylococcus aureus* (MRSA) were evaluated. MBC of HACC were determined by standardized broth microdilution techniques with inoculums of 5×10^5 CFU/mL according to Clinical Laboratory Standards Institute guidelines and incubated at 35 °C for 24 h [25].

2.2 Cytotoxicity testing of HACC

MC3T3-E1 cells were cultured in alpha-minimal essential medium (α-MEM, Invitrogen Co. CA) containing 10% fetal bovine serum (FBS) at 37°C in a CO_2 incubator (5% CO_2/95% air). MC3T3-E1 cells were sub-cultured at approximately 80% confluence in every 3 days in 75cm^2 flasks.

The cytotoxicity of HACC to MC3T3-E1 cells was assessed using Alamar Blue Assay (Invitrogen, CA, USA). MC3T3-E1 cells were seeded at 6×10^4 cells/well in 12-well plates. After 24 hours of incubation, the culture medium was changed into α-MEM culture medium containing 1000, 200, 40μg/mL of HACC. Alamar Blue was also added to each well in an amount equal to 5% (v/v) of the culture medium volume. Cells treated with a-MEM without HACC served as negative control. The supernatants of each well were extracted after 6, 24, 48 and 72 hours, and their fluorescence was measured with excitation at 530 nm and emission at 590 nm.

2.3 Effect of HACC on BMP2-induced differentiation of pre-osteoblasts

To investigate the influence of HACC on BMP2-induced differentiation of MC3T3-E1 cells, they were seeded at 1×10^5 cells/well in 12-well plates. The cells were firstly treated with or without 40μg/mL HACC for three days. Subsequently, culture medium was changed to a-MEM containing 1% FBS with or without 50ng/mL BMP2. Accordingly, four groups were established as follows:

Groups	Treated with 40 μg/mL HACC for 3 days	Treated with 50ng/ml BMP2
+HACC+BMP2	+	+
+HACC-BMP2	+	-
-HACC+BMP2	-	+
-HACC-BMP2	-	-

Alkaline phosphatase (ALP) activity and Osteocalcin (OCN) expression was assessed after BMP2 treatment for 4 and 7 days. At each time point, supernatants of each well were harvest to detect OCN level using the OCN enzyme linked immunosorbent assay (ELISA)

kits (Cloud-clone, Houston, TX, USA). Subsequently, cells were rinsed three times with PBS. 400μL of distilled water was then added to each well to obtain cell debris for ALP activity by using a p-nitrophenyl-phosphate colorimetric assay. The total protein was determined with a bicinchoninic acid assay (BCA assay) protein assay reagent kit (Pierce, Rockford, IL, USA).

To assess mineralized modules formation, alizarin red S staining was used after BMP2 treatment for 28days. The substrates were washed three times with PBS, fixed in 4% formaldehyde for 15 min and stained with 0.5 mL/well Alizarin Red Stain Solution for 20 min at room temperature. The cell monolayers were then washed with distilled water until the runoff ran clear. The images were produced using an optical microscope.

2.4 Fabrication of BioCaP and BMP2-BioCaP granules

BioCaP was fabricated by refining a well-established biomimetic mineralization approach [15]. A CaP solution (200 mM HCl, 20 mM $CaCl_2 \cdot 2H_2O$, 680 mM NaCl, and 10 mM Na_2HPO_4) buffered by TRIS (250 mM) to a pH of 7.4. The whole solution was incubated in a shaking water bath (50agitations/min) at 37°C for 24 hours. Thereafter all precipitations were retrieved and gently washed by PBS, strongly filtered and compressed to a block using a vacuum exhaust filtering method with a vacuum filter (0.22-μm pore, Corning, NY, USA) and an air pump. After drying in air circulation at room temperature overnight, the hardened block was ground and filtered with metallic mesh filters to obtain granules with a size of 0.25-0.5mm. The calcium phosphate solution was filtered with the vacuum filter (0.22μm pore) before buffering for sterilization. All the following procedures were performed under aseptic conditions.

BMP2 (INFUSE® Bone Graft, Medtronic, USA) can be introduced into the calcium phosphate solution at a final concentration of 2μg/ml before buffering as mentioned above and thereafter was co-precipitated into the interior of BioCaP to form BMP2-BioCaP.

2.5 Fabrication and characterization of BMP2-BioCaP/HACC complex

HACC powder was purchased from Lvshen Bioengineering Co., Ltd. (Jiangsu, China) and sterilized by gamma rays from a cobalt-60 source (Synergy Health, Ede, the Netherlands). HACC powder was dissolved into distilled water to prepare HACC solution, 30μL of which was thoroughly adsorbed on the surface of 100uL BioCaP-BMP2 to obtain one sample of the BMP2-BioCaP/HACC complex (60μg BMP2 for per sample). The BMP2-BioCaP/HACC complex carrying various amounts of HACC was fabricated to optimize the HACC content of each sample. The BMP2-BioCaP/HACC complex carrying 20μg, 4μg or 0.8μg was respectively named as BMP2-BioCaP/HACC-20, BMP2-BioCaP/HACC-4,

BMP2-BioCaP/HACC-0.8.

The surface characterization of the BMP2-BioCaP and BMP2-BioCaP/HACC complex was evaluated by scanning electron microscope (SEM) (XL30, Philips, the Netherlands). For this purpose, samples of the material were mounted on aluminum stubs and sputtered with gold particles to a thickness of 10-15nm.

2.6 Release kinetics of HACC and BMP2 from the BMP2-BioCaP/HACC complex

To monitor the release kinetics, six samples of fluorescence-labeled BMP2-BioCaP/HACC complex (100uL/sample) were incubated in 1mL 0.9% phosphate-buffered saline (PBS) at 37°C, pH 7.4 for up to 30 days with mild shaking (50 agitations/min). At each time point (hour 3, 6, day1, 2, 3, 5, 10, 15, 20, 25, 30), triplicate 100μl aliquots of the medium (containing released FITC-BSA and rhodamine B) were collected and replaced with fresh PBS. Fluorescence densities of FITC-BSA and rhodamine B were measured in a spectrophometer (Spectramax M2 Molecular Devices, CA, USA) (FITC-BSA: excitation wavelength at 485 nm and emission wavelength at 528 nm; rhodamine B: excitation wavelength at 530 nm and emission wavelength at 590 nm). Fluorescence readings were converted to amounts of protein using a standard curve, which was generated by preparing a dilution series of FITC-BSA or rhodamine B in PBS. The temporal release of FITC-BSA was expressed as a percentage of the total amount that had been incorporated into BioCaP granules. Likewise, the temporal release of rhodamine B was expressed as a percentage of the total amount that had been adsorbed on the surfaces of BioCaP granules.

2.7 Animal experiment

To evaluate osteoinductivity of BMP2-BioCaP/HACC complex and optimize the HACC concentration *in vivo*, six groups were established (n=6 samples per group): 1) BMP2-BioCaP; 2) BioCaP/HACC; 3) BioCaP; 4) BMP2-BioCaP/HACC-20; 5) BMP2-BioCaP/HACC-4; 6) BMP2-BioCaP/HACC-0.8. Each sample contains 100uL BMP2-BioCaP/HACC complex. We used dorsal subcutaneous pockets in Sprague-Dawley rats (12 weeks and weighing~500g) as the animal model. Two samples per rat were randomly implanted into (one on the left side and one on the right) the subcutaneous pockets and be trapped therein by suturing the incision site. The animal study adhered to the ethic laws and regulations of China and was approved by the Ethical Committee of Zhejiang Chinese Medical University.

2.8 Histological preparation and histolomorphometrical analysis

After 5 weeks, the samples with surrounding tissues were retrieved and fixed chemically,

and embedded in methylmethacrylate (MMA) as previously reported [26]. By applying a systematic random sampling strategy [27], samples were sawn into 6 or 7 slices of 600μm thickness 400μm apart. Slices of each sample were mounted on Plexiglas holders and polished. They were then stained with McNeal's tetrachrome, basic fuchsine, and toluidine blue O. Images of 10 random areas from each slide were recorded under a microscope (Nikon-Eclipse) at final magnification of 200 times for histomorphometric analysis. The volume density of tissues and materials were determined stereologically using the point counting technique [28]. The volume density of newly formed bone was calculated as their volume divided by the corresponding reference volume of each sample. The space under the fibrous capsule that embraced the whole block of implants (subcupsullar space) was taken as the reference volume. The volume density of the remaining BioCaP granules was calculated as the absolute volume of the remaining BioCaP granules divided by the original volume of the BioCaP granules (100μL).

2.9 Statistical analysis

All data were presented as the mean values with the standard deviation (mean ± standard deviation). Data were compared using a one-way variance analysis (ANOVA) of with the significance level being set at $p < 0.05$. Post hoc comparisons were made using Bonferroni corrections.

3. **Results**

3.1 Determination of the optimal concentration of HACC

To determine the ideal point of balance between cellular biocompatibility an antibacterial performance of HACC, the influence of HACC concentration on viability of MC3T3-E1 cells was evaluated. The results (Fig.1) revealed that HACC at 200μg/mL and above could significantly suppress cell viability, whereas 40μg/mL HACC did not exhibited cytotoxicity to cells. What's more, the MBC of HACC against MRSA was 40μg/mL. 40μg/mL HACC was therefore considered as the optimal concentration of HACC to kill bacteria without harming osteoblasts, which was used for the following experiments.

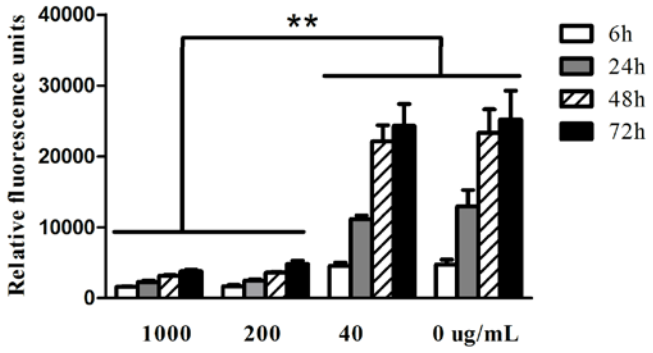

Fig 1. Cell viability of MC3T3-E1 after 6, 24, 48, 72h exposure to HACC at concentrations of 1000, 200, 40 and 0μg/mL. Mean values are presented together with the standard deviation. *p<0.05.

3.2 The influence of HACC on BMP2-induced cell differentiation

According to our pilot study, HACC was resorbed within 3 days *in vivo*. To mimic the *in-vivo* situation, MC3T3-E1 cells were firstly treated with HACC at its optimal concentration-40μg/mL for 3 days. After removal of HACC, cells were then exposed to BMP2. ALP and OCN, markers of osteoid formation and bone mineralization, were used to examine the influence of HACC on BMP2-induced cell differentiation. The ALP assay (Fig. 2A) and OCN assay (Fig. 2B) showed that when cells were not pre-treated with HACC, BMP2 significantly improved ALP and OCN expression of cells at 4 days and 7 days. When cells were pretreated with 40μg/mL HACC, the ALP and OCN expression of them was also improved with exposure to BMP2. Moreover, the ALP and OCN expression showed no significant difference between group +HACC+BMP2 and –HACC+BMP2. Besides, mineralized modules formation was found in group +HACC+BMP2 and –HACC+BMP2. These results indicate that the BMP2-induced cell differentiation were not influence by pretreatment of HACC or not.

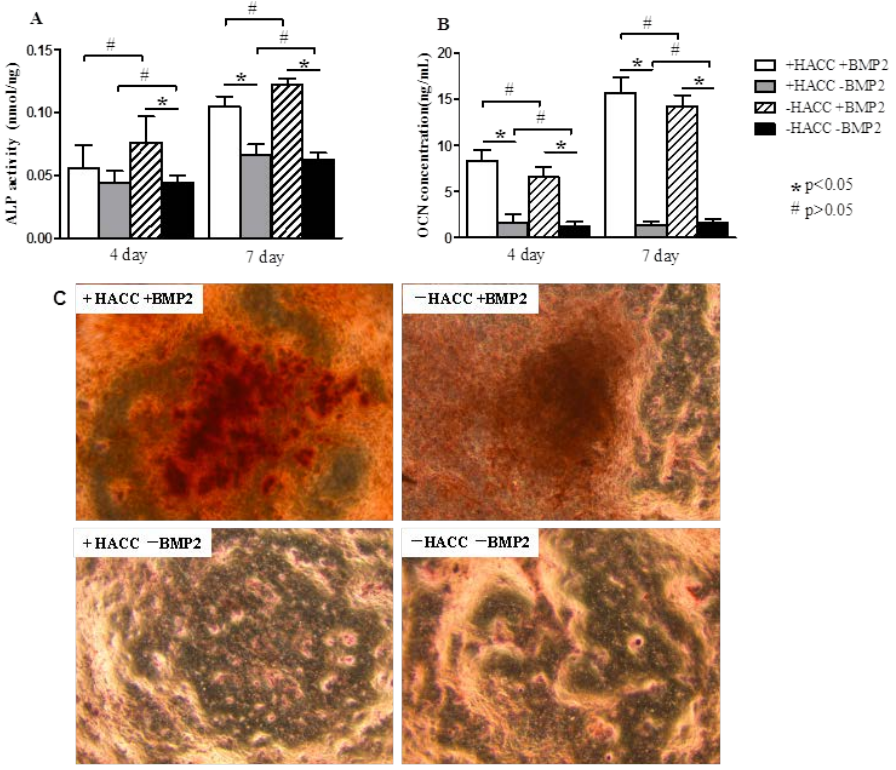

Fig 2. The ALP activity (A) and OCN concentration (B) of MC3T3-E1 cells after treatment of HACC and/or BMP2 for 4 and 7 days. Mean values are presented together with the standard deviation (* p<0.5; # p>0.5). Alizarin red staining (C) on MC3T3-E1 cells with 3-days pre-treatment of HACC and following treatment of BMP2 for 28 days. 100× magnification.

3.3 Fabrication and characterization of the BMP2-BioCaP/HACC complex

As 40μg/mL has been confirmed as the optimal concentration of HACC, we therefore need to assure the *in-vivo* functioning concentration of HACC should be 40μg/mL. We assumed that *in vivo* HACC would release from the BMP2-BioCaP/HACC complex and functioned locally in a niche around the BMP2-BioCaP/HACC complex where HACC concentration was 40μg/mL. Given that the volume of each BMP2-BioCaP/HACC complex is 100μL, we speculated the niche around the BMP2-BioCaP/HACC complex should be around 100μL. 500, 100, 20μL were therefore taken. Accordingly, the HACC amount carried by each BMP2-BioCaP/HACC complex sample was 20, 4 and 0.8μg, which were respectively named as BMP2-BioCaP/HACC-20, BMP2-BioCaP/HACC-4, BMP2-BioCaP/HACC-0.8. The size of BMP2-BioCaP granules ranged from 0.25- 0.50 mm. The SEM images of

BMP2-BioCaP/HACC showed that BMP2-BioCaP granules were embedded into the HACC network.

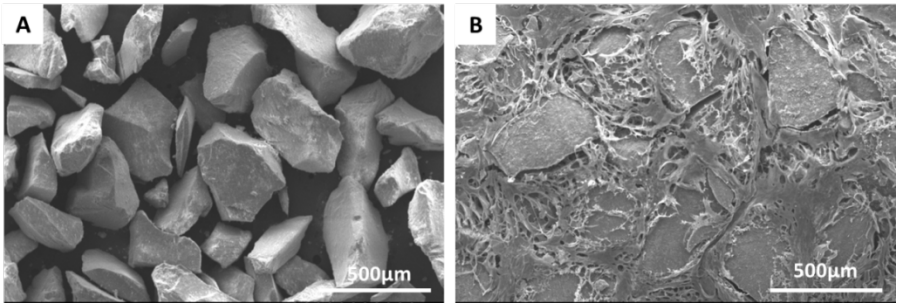

Fig 3. SEM micrographs of BMP2-BioCaP (A) and BMP2-BioCaP/HACC complex (B).

3.4 Release kinetics of HACC and BMP2 from the BMP2-BioCaP/HACC complex

The *in-vitro* release profile (Fig. 3) showed that HACC was therefore released rapidly and completely exhausted after 3 days, while BMP2 revealed a low burst release and subsequently a sustained release from the internally-incorporated depot in BioCaP granules. About 10-14% of BMP2 released from BioCaP granules in an initial burst release stage (within in the first 24 hours). It was then gradually released at a steady rate until 30 days.

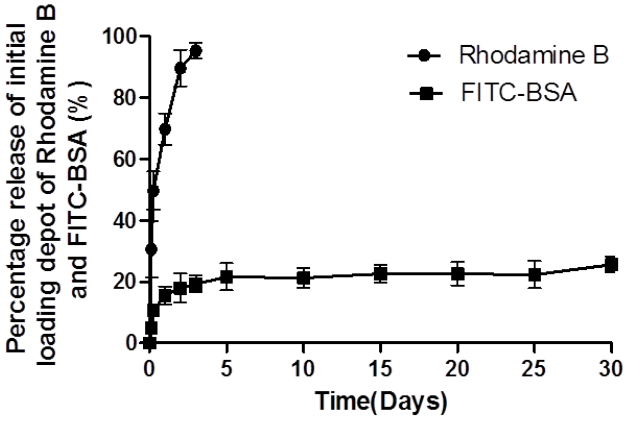

Fig 4. Graph depicting the cumulative release kinetics of rhodamine B (HACC substitute) and FITC-BSA (BMP2 substitute) from BMP2-BioCaP/HACC complex. Mean values are presented together with the standard deviation.

3.5 Ectopic bone formation of BMP2-BioCaP/HACC complex

After initial optimization on HACC content of each the BMP2-BioCaP/HACC complex sample *in vitro*, the ectopic bone formation of the BMP2-BioCaP/HACC complex in dorsal subcutaneous pockets of rats were used to evaluate the osteoinductivity of the BMP2-BioCaP/HACC complex *in vivo*. The light microscope of representative histological sections of each group at 5 weeks (Fig. 5) showed that newly formed bone was deposited directly upon the surface of BioCaP granules, and was only found in BMP2-containing groups. According to histomorphometrical analysis (Fig. 6), in comparison with the positive control group (BMP2-BioCaP), BMP2-BioCaP/HACC-4 and BMP2-BioCaP/HACC-0.8 complex resulted in similar amount of new bone formation, while BMP2-BioCaP/HACC-20 was found significantly less new bone formation and less mature bone structure (Fig. 5D). In group of BMP2-BioCaP/HACC-4, we observed that compact bone area in an active phase with osteoblasts (Fig. 5E). On the other hand in groups without BMP2, no bone tissue but fibrous capsular tissue was found around embracing the BioCaP granules (Fig. 5B&C).

HACC could not be observed on histological sections any more after 5 weeks (Fig. 5).

The degradation of BioCaP with or without BMP2 did not exhibit significant difference among groups, indicating the existence of HACC did not influence BioCaP degradation in a pro-fibrotic environment (Fig. 6B).

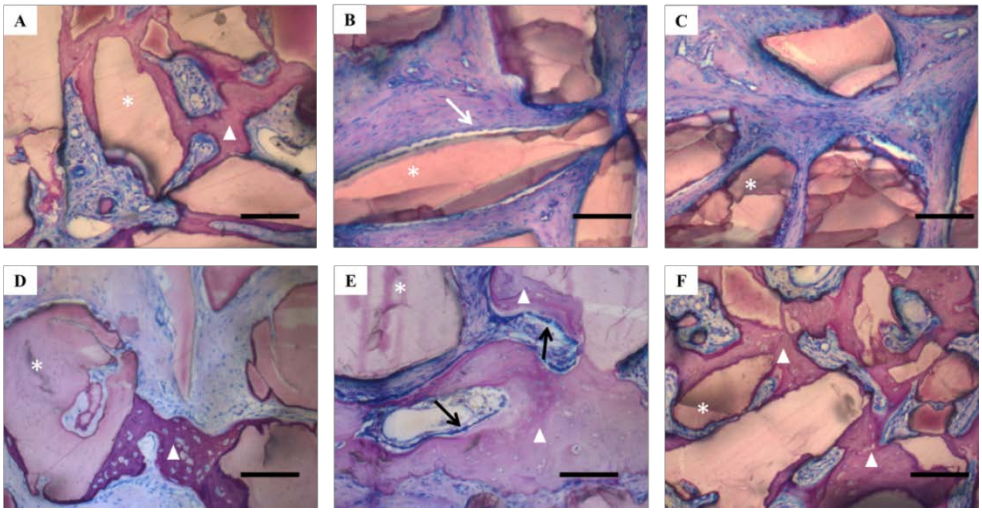

Fig 5. Light microscope of representative histological sections of each group at 5 weeks after subcutaneous implantation in rats: A) BMP2-BioCaP; B) BioCaP/HACC; C) BioCaP; D) BMP2-BioCaP/HACC-20; E) BMP2-

BioCaP/HACC-4; F) BMP2-BioCaP/HACC -0.8. The sections were stained with McNeal's Tetrachrome, basic Fushine and Toludine Blue O. Newly formed bone (white arrow head)was deposited directly upon the surface of BioCaP granules (asterisks), and was only found in BMP2-containing groups (A,D, E and F). New bone tissue in group D exhibited less mature structure and smaller quantities that in A. Compact bone area in an active phase with osteoblasts (black arrow) was observed in groups E. In groups without BMP2 (B&C), no bone tissue but fibrous capsular tissue (white arrow) was found around embracing the BioCaP granules. Scale bar=100μm.

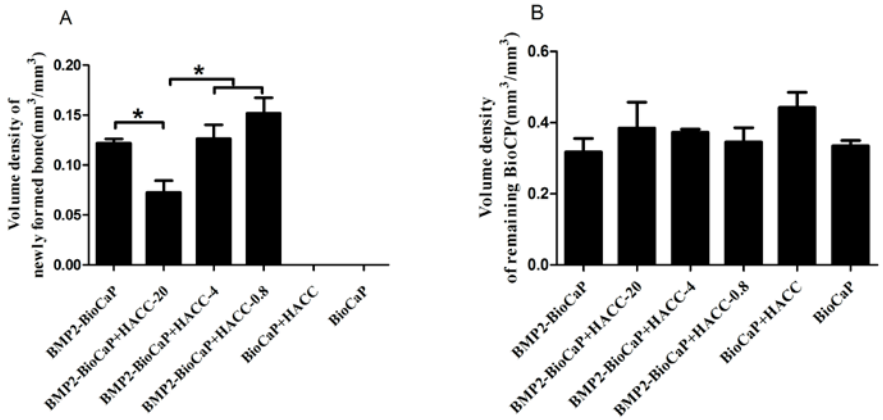

Fig 6. Graphs depicting the volume of newly formed bone (A) and the volume of remaining BMP2-BioCaP/BioCaP granules (B) 5 weeks after implantation. Mean values are presented together with the standard deviation. *p<0.05.

4. Discussion

Treatment of infected critical-sized bone defects remains a great challenge of orthopedic surgery, oral and maxillofacial surgery because of the local residual bacteria and beyond-self-healing bone defects. To overcome these two difficulties and consequently repair the infected critical-sized bone defects, we believe the ideal local biomaterial system should meet the following requirements: 1) The biomaterial system can function as both antibiotic carrier and bone substitute, to not only clear the infection but to also contribute to the subsequent bone regeneration process; 2) The antibiotics used for local delivery should have a broad spectrum of activity and a low rate of bacteria resistance; 3) The antibiotic should also be delivered to its optimal concentration, at which it reaches the balance between cellular toxicity and antibacterial activity; 4) Osteoinductive bone grafts are more favorable for improving bone regeneration; 5) The release kinetics of antibiotic and osteoinductive agents should meet their optimal delivery mode respectively.

Firstly, the application of current available local biomaterial system is limited due to their

intrinsic drawbacks. PMMA bone cement is considered as the gold standard biomaterial for local antibiotic therapy. PMMA bone cements containing either gentamicin or tobramycin at low doses (0.5-1 g per 40 g cement), are sufficient products for prophylaxis, but not for therapeutic applications in established osteomyelitis [29]. In addition to the limitations on antibiotic choice and the elution kinetics and efficiency, the other major shortcoming of PMMA is that it is non-biodegradable. Consequently, it must be removed after infection management as it could impair healing of the debrided bone defect. To preclude the issue of biodegradation, calcium sulfate is developed as the primary resorbable material that has been used clinically for local antibiotic delivery. On average, calcium sulfate pellets take approximately 2-3 months to radiographically resorb [30]. These calcium-based antibiotic-delivery systems have advantages over PMMA in that they can carry a wider range of antibiotics and do not need a second surgery to remove them. However, for critical-sized bone defect cases, after the removal or resorption of these local antibiotic-delivery systems, a second surgery is still needed for bone grafting. The drawback of this two-step surgical approach, however, is that it requires considerable time and additional surgeries, which increase treatment costs and patient burden. Therefore, development of novel local biomaterial system has primarily focused on biodegradable materials which can function as both antibiotic carrier and bone substitute. Based on this theory, we used our previously developed osteoinductive bone substitute, BMP2-BioCaP granules, also as antibiotic carrier to develop an antibacterial and osteoinductive biomaterial─the BMP2-BioCaP/HACC complex. It could also rapidly kill site-infection associated bacteria, and then induce bone formation in profibrous environment (Fig. 5), which is as challenging as in critical-sized bone defects. The complex was observed being incorporated into newly formed bone and showing the trend of being degraded eventually. Compared to two-step surgical approach, the benefit of using the BMP2-BioCaP/HACC for treating infected critical-sized bone defects is that it only needs one surgery, which can dramatically decrease treatment costs and patient burden.

Secondly, the antibiotics used in the local biomaterial system plays a key role for the infection control. Since a wide of Gram-positive (e.g. *S. aureus* and *S. epidermidis*) and Gram-negative (e.g. *Pseudomona aeruginosa* and *Escherichia coli*) bacteria are found in the infected defects [18], the biomaterial system should have a broad-spectrum antibiotic. Vancomycin, one of the most commonly used antibiotics in local delivery system, is only active against Gram-positive bacteria such as *Staphylococci* (including MRSA), *Streptococci and Enterococci* [31]. It is therefore not an ideal choice for local infection control. In contrast, another most commonly used antibiotic─gentamicin has a broad-spectrum of activity, rapid concentration-dependent bactericidal effect and low cost [32]. It is available for intramuscular and intravenous injection, in antibiotic impregnated PMMA beads, in sponge-like collagen implants and as antibiotic component in a coating on intramedullary nails for tibial fracture fixation [18]. However, it cannot be the perfect

antibiotic for the local biomaterial system due to the more and more severe bacterial resistance. As a result of indiscriminate use of these conventional antimicrobials, the spread of antimicrobial resistance is now a global problem. The emergence of resistant strains has increased the morbidity and mortality associated with wound infections [33]. Therefore, research has also focused on encapsulating other novel antimicrobial compounds into biomaterials, such as modified antibiotics, silver or antimicrobial peptides. HACC, a new water-soluble quaternary ammonium salts from the reaction of chitosan with glycidyl trimethylammonium chloride, have been reported as a chitosan derivative with a broad spectrum of antibacterial ability [34]. The mechanism of antibacterial action of HACC may explain its broad spectrum. Despite the distinction between Gram-positive and Gram-negative bacterial cell walls, antibacterial modes both begin with interactions at the cell surface and compromise the cell wall first. Gram-positive bacteria, lipoteichoic acids may provide a molecular linkage for HACC at the cell surface, allowing it to disturb membrane functions [35]. Lipopolysaccharides and proteins in the Gram-negative bacteria outer membrane are held together by electrostatic interactions with divalent cations that are required to stabilize the outer membrane. Polycations may compete with divalent metals, such as Mg^{2+} and Ca^{2+} ions present in the cell wall, which will disrupt the integrity of the cell wall or influence the activity of degradative enzymes [36]. Therefore, HACC shows antibacterial activity to both Gram-positive and Gram-negative bacteria.

Furthermore, there is very few report of the bacteria resistance of HACC, which is perhaps because it can inhibit biofilm formation. The presence of bacteria in a biofilm drastically reduces their susceptibility to antimicrobial drugs and host defense cells. For this reason, it is better to prevent biofilm formation rather than trying to kill bacteria after they are shielded by biofilm. The first step of biofilm formation is bacterial adherence to orthopedic implant surfaces [37]. Its adherence to the implanted surface is followed by an accumulation process and the production of extracellular substances. Researchers found that HACC could inhibit bacteria in expressing the intercellular adhesin, a kind of extracellular substance, so as to inhibit biofilm formation.

Thirdly, many antibiotics are naturally associated with the issue of their cytotoxicity [38, 39], it was therefore of great importance to find the optimal HACC concentration to effectively kill bacteria without harming cells and tissues so as to guarantee the two equally important properties of the BMP2-BioCaP/HACC complex─antibacterial activity and osteoinductivity *in vitro* and *in vivo.* In the present study, the proliferation of rat pre-osteoblast MC3T3-E1 cells and antibacterial efficacy for different concentrations of HACC were evaluated to strike a balance between cellular cytotoxicity and antibacterial efficacy (Fig. 1). As 40μg/mL has been confirmed as the optimal concentration of HACC, we need to assure the *in-vivo* functioning concentration of HACC is also 40μg/mL. We assumed that *in vivo* HACC would release from the BMP2-BioCaP/HACC complex and functioned

locally in a niche around it where HACC concentration was 40μg/mL. Given that the volume of each BMP2-BioCaP/HACC complex is 100μL, we speculated the niche around the BMP2-BioCaP/HACC complex should be around 100μL. Volume around 100μL, such as 500, 100, 20μL, were therefore taken to verify how wide HACC can spread in vivo. Accordingly, the HACC amount carried by each BMP2-BioCaP/HACC complex sample was 20, 4 and 0.8μg, which were respectively named as BMP2-BioCaP/HACC-20, BMP2-BioCaP/HACC-4, BMP2-BioCaP/HACC-0.8. The *in-vivo* results of osteoinductivity (Fig. 6A) revealed that in comparison with the positive control group (BMP2-BioCaP), BMP2-BioCaP/HACC-4 and BMP2-BioCaP/HACC-0.8 complex resulted in similar amount of new bone formation, while BMP2-BioCaP/HACC-20 was found significantly less newly formed bone and less mature bone structure. It indicated that in the niche around BioCaP/HACC-20, the concentration of HACC was higher than 40μg/mL, and consequently showed certain bone formation suppression. This phenomenon suggested HACC would not spread as widely as 5 times wider than its own volume and confirmed our assumption that HACC released from the BMP2-BioCaP/HACC complex and functioned locally in a niche around it.

Fourthly, in the progress of bone regeneration, growth factors are needed to differentiate progenitor cell into osteoblasts and subsequently to form new bone. Based on this theory, there is an increasing interest in combining osteogenic and/or vasculogenic growth factors with osteoconductive materials to enhance bone regeneration. This kind of materials is considered to possessing osteoinductivity, which is defined as the ability to induce progenitor cells to differentiate down osteogenic lineages [13]. The golden proof of osteoinductivity of certain biomaterial is that it form bone tissue in ectopic site, such as subcutaneous pockets of rats [40]. As shown in figure 5 and 6A, compared to BMP2-free BioCaP granules, the BMP2-BioCaP/HACC complex could form bone tissue in the ectopic sites, which confirmed its osteoinductivity and its capacity of enhance new bone formation.

BMP2 is an effective, but expensive osteoinductive agent. An ideal BMP2-carrier should consume BMP2 efficiently. In current study, 100μL BMP2-BioCaP/HACC complex, carrying only 60μg BMP2, successfully gain bone tissue in a profibrous environment. Compared to milligram of BMP2 used in previous study [22], only 60μg BMP2 can achieve bone formation in the profibrous environment, which indicated that the BMP2-BioCaP/HACC complex delivered BMP2 efficiently. Moreover, BMP2 is internally incorporated into the BMP2-BioCaP/HACC complex. This could facilitate the retention of BMP2 at the site of implantation for a prolonged period and protect the integrity of BMP-2 against interstitial proteases [41], eventually enhancing the osteoinductive capacity of the complex.

Fifthly, sequential release of antibiotic and osteoinductive agent also guarantees the success

of the BMP2-BioCaP/HACC complex in repairing infected bone defects. As shown in *in-vitro* release kinetic assay (Fig. 4), the burst delivery of HACC was followed by slow and sustained release of BMP2 at its low concentration, which was in line with the optimal delivery mode of both HACC and BMP2 as mentioned above.

The burst release of HACC was designed to rapidly kill residual bacteria in infected bone defects without resulting in bacterial resistance. Data emerging from *in-vitro* and *in-vivo* studies suggested that inappropriately low antibiotic dosing may be contributing to the increasing rate of antibiotic resistance [42]. We speculated that long-term, low-dosage of antibiotics offered enough time for resistance strains being selected and significantly proliferated, therefore it is reasonable to avoid resistance by burst release a strong antibiotic to kill bacteria rapidly without giving bacteria time to proliferate. Since HACC, the strong antibiotics, was absorbed on the surface of BMP2-BioCaP, once BMP2-BioCaP/HACC complex was implant *in vivo*, it could rapidly dissolve into body fluid to achieve burst release so as to kill bacteria. Moreover, as mentioned above, the ability of HACC in inhibiting biofilm formation also contributed to avoid resistance.

To function as an effective osteoinductive agent, BMP2 needs to be released slowly and continuously at its low concentration [14]. Based on this theory, we developed BMP2-BioCaP granules [15] where BMP2 was internally incorporated into the BioCaP granules. BMP2 was therefore released slowly and continuously with the undergoing degradation of BioCaP granules *in vivo*. This has been indirectly proved by our previous study (Unpublished data) revealing the parallel release kinetics of BMP2 and Ca^{2+} from BMP2-BioCaP. The bone formation (Fig. 5) observed in the BMP2-BioCaP/HACC complex also supports that it reached the optimal delivery mode of BMP2.

In the present study, the feasibility of the BMP2-BioCaP/HACC complex applied in bone tissue engineering was assessed. The current results suggested that the BMP2-BioCaP/HACC complex is particularly suitable to repair infected bone regeneration due to rapid killing of residual bacteria and effective promotion of osteogenesis. However, the present study has notable limitations. Because the pathogenesis of infections in infected bone defects is complex process and involves interactions between the pathogen, biomaterial, and host, the *in-vitro* assays discussed here do not account for host defense and some other *in-vivo* factors. Moreover, although ectopic bone formation is the gold standard to confirm the osteoinductivity of a material, the animal model used in the present study cannot fully represent bone-defect model. In our ongoing studies, *in-vivo* infected-bone-defect models will be utilized to evaluate the antibacterial efficacy of BMP2-BioCaP/HACC complex.

5. **Conclusion**

In this study, we developed a novel antibacterial and osteoinductive BMP2-BioCaP/HACC complex with sequential release of antibiotic and osteoinductive agent—short-term delivery of HACC and following slow and sustained release of BMP2. It could rapidly eliminate antibiotic-resistant bacteria and efficiently promote new bone formation both *in vitro* and *in vivo*, which conferred this novel material a promising application potential to repair infected critical-sized bone defects. In our ongoing studies, *in-vivo* infected-bone-defect models will be utilized to evaluate the antibacterial efficacy of BMP2-BioCaP/HACC complex.

6. References

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