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ORIGINAL ARTICLE

In Situ Gelling Hydrogel with Anti-Bacterial Activity and Bone Healing Property for Treatment of Osteomyelitis

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Abstract

BACKGROUND: Despite the development of progressive surgical techniques and antibiotics, osteomyelitis is a big challenge for orthopedic surgeons. The main aim of this study is to fabricate an *in situ* gelling hydrogel that permits sustained release of antibiotic (for control of infection) and growth factor (for induction of new bone formation) for effective treatment of osteomyelitis.

METHODS: An *in situ* gelling alginate (ALG)/hyaluronic acid (HA) hydrogel containing vancomycin (antibiotic) and bone morphogenetic protein-2 (BMP-2; growth factor) was prepared by simple mixing of ALG/HA/Na_2HPO_4 solution and $CaSO_4$/vancomycin/BMP-2 solution. The release behaviors of vancomycin and BMP-2, anti-bacterial effect (*in vitro*); and therapeutic efficiency for osteomyelitis and bone regeneration (*in vivo*, osteomyelitis rat model) of the vancomycin and BMP-2-incorporated ALG/HA hydrogel were investigated.

RESULTS: The gelation time of the ALG/HA hydrogel was controlled into approximately 4 min, which is sufficient time for handling and injection into osteomyelitis lesion. Both vancomycin and BMP-2 were continuously released from the hydrogel for 6 weeks. From the *in vitro* studies, the ALG/HA hydrogel showed an effective anti-bacterial activity without significant cytotoxicity for 6 weeks. From an *in vivo* animal study using Sprague–Dawley rats with osteomyelitis in femur as a model animal, it was demonstrated that the ALG/HA hydrogel was effective for suppressing bacteria (*Staphylococcus aureus*) proliferation at the osteomyelitis lesion and enhancing bone regeneration without additional bone grafts.

CONCLUSIONS: From the results, we suggest that the *in situ* gelling ALG/HA hydrogel containing vancomycin and BMP-2 can be a feasible therapeutic tool to treat osteomyelitis.

Keywords Osteomyelitis · Hydrogel · Vancomycin · BMP-2 · Anti-bacterial activity · Bone regeneration

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1 Introduction

Osteomyelitis, which is defined as a bacterial infection of bone or bone marrow, is a challenging problem for orthopedic surgeons [1, 2]. It is well-established that artificial hip/knee joint replacement, bone fracture with environmental exposure, bone surgery, and soft tissue infection are major risk factors of the ailment [3, 4]. Although healthy human bone is highly resistant to infection [5], the

incidence rate of osteomyelitis is gradually increased by increase in the aging population with osteoarthritis, rheumatoid arthritis, osteoporosis, and immunocompromised symptom [4, 6]. The bacteria in the lesion releases osteolytic cytokines and osteonecrosis factors, and this leads to severe pain and bone loss associated with devascularization [3, 7, 8]. Therefore, a two-step treatment of surgical debridement of infected bone/systemic and/or local delivery of antibiotics for control of infection and the subsequent implantation of bone graft through additional surgery for reconstruction of bone has been adapted as a gold standard therapy for osteomyelitis [9]. In recent years, a local delivery system based on antibiotic-containing synthetic poly (methyl methacrylate) (PMMA) bone cement [10, 11] has been used more widely in clinical practice compared with a systemic delivery system, because it can ensure suitable antibiotic concentration at the disease site despite limited vascularity, and prevent systemic toxicity of high doses of antibiotic (i.e., nephrotoxicity, ototoxicity, gastrointestinal side effects, etc.) [12–15]. Various antibiotics including vancomycin, gentamycin, tobramycin, cefuroxime, erythromycin, and colistin have been used for antibiotic-containing PMMA bone cements [16]. However, residual monomers in the PMMA matrix which lead to necrosis of surrounding bone [17]; fast (within few days) and partial (< 10% of loading amount) release of the antibiotic incorporated in the PMMA matrix which does not guarantee effectiveness of antibiotic and rather stimulates antibiotic resistance [18–20]; and the necessity of an additional surgery to eliminate the non-degradable matrix that causes another infection [21] remain as inevitable problems in clinical fields. To compensate for the limitations of the PMMA-based antibiotic carrier system, antibiotic-incorporated matrices including hydroxyapatite [22], tricalcium phosphate [23], calcium sulfate [24], polycaprolactone (PCL) [25], poly(lactic-*co*-glycolic acid) (PLGA) [14, 26], fibrin glue [27], collagen [28], and cross-linked hyaluronic acid [29] have been extensively studied in laboratories and clinics. However, to the best of our knowledge, there are few reports on matrix system for both effective control of infection and sufficient bone regeneration without additional operations. Based on the literature, we hypothesized that if a bioabsorbable matrix to provide continuous release of antibiotic for the treatment of infection and bioactive molecule for the induction of new bone formation for a sufficient period could be developed, it would be a promising therapeutic system for the treatment of osteomyelitis to reduce the number of surgeries, shorten the treatment period, and thus relieve a patient's physical/economic burden.

In our previous study [30], we reported that *in situ* gelling alginate (ALG)/hyaluronic acid (HA) hydrogel provides sustained release of bioactive molecule [i.e., bone morphogenetic protein-2 (BMP-2)] and thus allows enhanced bone regeneration. Gelation rate of ALG/HA hydrogel was controlled by adjusting the ratio between calcium sulfate ($CaSO_4$, crosslinking agent of ALG) and disodium hydrogenphosphate (Na_2HPO_4, crosslinking retardation agent). HA exhibits osteoinductive and proangiogenic properties and has gained increasing interest as a biomedical material inducing bone regeneration [31]. The BMP-2 with osteogenic [32] and angiogenic [33] activities was entrapped in the ALG/HA hydrogel and was slowly released from the hydrogel by diffusion through the network structure in the hydrogel. In this study, we prepared an *in situ* gelling ALG/HA hydrogel containing antibiotic and bioactive molecule that can provide continuous release of the additives for an adequate period of time for fundamental treatment of osteomyelitis without additional surgery to remove the implanted matrix and implant bone grafts (Fig. 1). A positively charged vancomycin was selected in the study because it leads to charge–charge interaction with negatively charged ALG; thus sustained release from the ALG/HA hydrogel may be achieved despite the hydrophilic characteristics of the drug [34]. Vancomycin is frequently used as an antibiotic for local delivery for osteomyelitis and is also considered as an antibiotic for the treatment of lesions infected with methicillin resistant *Staphylococcus aureus* (MRSA) [35, 36]. The release behaviors of vancomycin and BMP-2, anti-bacterial effect (*in vitro*); and therapeutic efficiency for osteomyelitis and bone regeneration (*in vivo*, osteomyelitis rat model) of the vancomycin and BMP-2-incorporated ALG/HA hydrogel were investigated.

2 Materials and methods

2.1 Materials

Sodium alginate (ALG; medium viscosity; Sigma, St. Louis, MO, USA) was washed using methanol (65%) to purify [37]. Hyaluronic acid (HA; Mw 3000 kDa; SK Bioland, Cheonan, Republic of Korea), $CaSO_4$ (Oriental Chemical Industries, Seoul Republic of Korea), and Na_2HPO_4 (Junsei Chemical, Tokyo, Japan) were used without further purification. Vancomycin hydrochloride (VAN; Kukjeon Pharm, Anyang, Republic of Korea) and bone morphogenetic protein-2 (BMP-2; R & D Systems, Minneapolis, MN, USA) were selected as bioactive molecules for infection control and bone regeneration, respectively.

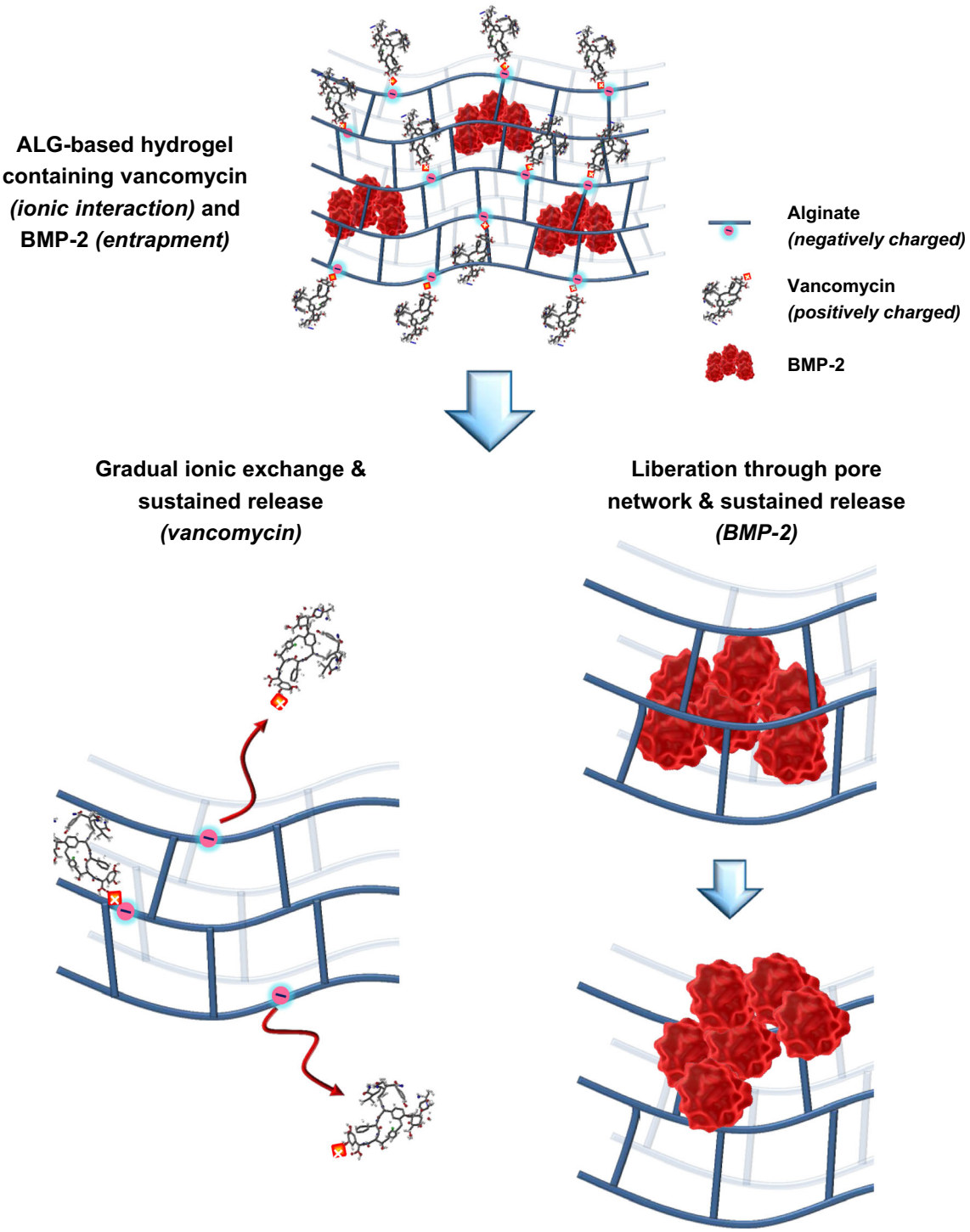

Fig. 1 Schematic diagram showing the possible mechanism for loading of vancomycin (ionic interaction) and BMP-2 (entrapment) in the *in situ* gelling ALG/HA hydrogel, and their continuous release in the body over time

2.2 Fabrication of vancomycin- and BMP-2-incorporated ALG/HA hydrogel

ALG and HA powder mixture [ALG/HA, 7/3 (w/w)] was dissolved in phosphate buffered saline (PBS; concentration of Na_2HPO_4, 0.11 wt%, pH ~ 7.4) to prepare a polymer concentration of 2 wt%. After $CaSO_4$ (0.8 wt%), vancomycin (each 60, 100, and 140 mg/mL), and BMP-2 (2 μg/mL) were evenly mixed in cold PBS (4 °C), 2 wt% ALG/HA solution was mixed with the same volume of the $CaSO_4$/vancomycin/BMP-2 solutions to form vancomycin- and BMP-2-incorporated ALG/HA hydrogel (*VAN/BMP-*

ALG/HA; final concentrations: ALG/HA (7/3), 1 wt%; $CaSO_4$, 0.4 wt%; VAN, each 30, 50, and 70 mg/mL; BMP-2, 1 μg/mL). The mixture solutions were injected into glass vial (for *in vitro* experiment) or defect site (for *in vivo* experiment), and kept for 4 min to allow stable gelation [38].

To compare the effect of *VAN/BMP-ALG/HA* hydrogel on infection control and bone regeneration, ALG/HA hydrogel (*ALG/HA*), vancomycin-incorporated ALG/HA hydrogel (*VAN-ALG/HA*), and BMP-2-immobilized ALG/HA hydrogel (*BMP-ALG/HA*) were also prepared using the same procedures as above.

2.3 Release test of vancomycin and BMP-2

To investigate the release pattern of vancomycin and BMP-2 from *VAN/BMP-ALG/HA* hydrogels (vancomycin concentrations, 30, 50, and 70 mg/mL; BMP-2 concentration, 1 μg/mL), *VAN/BMP-ALG/HA* hydrogels (1 mL) were incubated in PBS (1 mL in 5-mL glass vial) containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 6 weeks. At predesignated time intervals (1 and 3 days and 1, 2, 3, 4, 5, and 6 weeks), the whole medium in the vial was harvested and filled with fresh PBS with the same volume. The amount of released vancomycin and BMP-2 from the *VAN/BMP-ALG/HA* hydrogels at each time point was determined using a UV/VIS spectrometer (absorbance at 240 nm; UV-3600, Shimadzu, Kyoto, Japan) and ELISA kit (R & D Systems, Minneapolis, MN, USA), respectively.

To confirm the charge–charge interaction between negatively charged ALG and positively charged vancomycin, 50 mg vancomycin was dissolved in 2 wt% ALG solution or 27.5 wt% Pluronic F127 (neutral charge) solution (1 mL). Each solution was placed in a 2-mL glass vial and 1 mL of PBS was carefully added on the surface of the solutions. At predetermined time points (1, 3, 6, 12, 24, and 36 h), the whole medium was carefully harvested and filled with fresh PBS. The release behavior of vancomycin from each solution was compared through the same analysis procedures as above.

2.4 Cytotoxicity test

The cytotoxicity of vancomycin released from *VAN/BMP-ALG/HA* hydrogel was evaluated, according to the modified ISO 10993-5 (2009). The *VAN/BMP-ALG/HA* (1 mL) with different vancomycin concentration (30, 50, and 70 mg/mL; 30-mg *VAN/BMP-ALG/HA*, 50-mg *VAN/BMP-ALG/HA*, and 70-mg *VAN/BMP-ALG/HA,* respectively) was incubated in a 10 mL Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) at 37 °C for 24 h under mild shaking (50 rpm), and the whole medium containing vancomycin released from the hydrogel was collected. To investigate the cytotoxicity of the vancomycin in the harvested medium, murine calvaria pre-osteoblast (MC3T3-E1; CRL-2593, ATCC, Manassas, VA, USA) was used. The MC3T3-E1 cells in DMEM containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin G (Sigma, St. Louis, MO, USA), and 0.1% gentamicin sulfate (Sigma, St. Louis, MO, USA) were seeded into culture plates (96-well; Corning, Corning, NY, USA) at a density of 1×10^4 cells/well. After 1 day, the cell culture medium was exchanged with the harvested vancomycin-containing medium (0.2 mL, 10% FBS added). After 1 day incubation, the cell viability was measured by an MTS assay. The normal cell culture medium was used for a control group (cell viability, 100%).

2.5 *In vitro* anti-bacterial activity test

In vitro anti-bacterial activity of vancomycin released from *VAN/BMP-ALG/HA* hydrogel or free vancomycin was evaluated by a paper disk diffusion inhibition test. The *VAN/BMP-ALG/HA* with different vancomycin concentration (30, 50, and 70 mg/mL) or free vancomycin powder [30, 50, and 70 mg; 30-mg *free VAN*, 50-mg *free VAN*, and *70-mg free VAN, respectively*)] was incubated in 1 mL PBS at 37 °C for up to 6 weeks (required period for the treatment of osteomyelitis [39]) under mild shaking (~ 50 rpm). At predesignated time intervals (1, 7, 21, and 42 days for *VAN/BMP-ALG/HA*; 1 and 2 days for free *VAN*), the whole medium was harvested and filled with fresh medium. To investigate the anti-bacterial activity, *Staphylococcus aureus* (*S. aureus*) KCTC1621 (a predominant bacteria in osteomyelitis [40]) was used as a model microorganism. The *S. aureus* was inoculated on Petri-dishes (Corning, Coring, NY, USA) with nutrient agar (15 g agar, 5 g peptone, 3 g beef extract, and 1 L water), and sterile filter papers (diameter 8 mm and thickness 0.7 mm) wet with each collected medium (50 μL) were put on the Petri-dishes. Sterile filter papers impregnated with PBS or medium harvested from the *ALG/HA* group (using the same incubation procedure as above) were also used as control groups. The anti-bacterial activity of vancomycin released from the *VAN/BMP-ALG/HA* group and other groups was compared by zone of inhibition (ZOI). The ZOI was determined using an image analysis program (*i*-Solution, IMT, Daejeon, Republic of Korea).

2.6 *In vivo* anti-bacterial activity and bone regeneration evaluation

To investigate *in vivo* anti-bacterial activity and bone reconstruction behavior of *VAN/BMP-ALG/HA* hydrogel,

Sprague–Dawley (SD) rats with osteomyelitis in femur were used as a model animal. All animal studies were approved from the Animal Care Committee of Hannam University of Korea. A total of 30 rats were used for the experiments. The rats were divided into five groups as follows: normal group, osteomyelitis group (osteomyelitis femur), *ALG/HA* group (injected with ALG/HA hydrogel into the osteomyelitis femur), *VAN-ALG/HA* group [injected with vancomycin (50 mg/mL)-incorporated ALG/HA hydrogel into the osteomyelitis femur], and *VAN/BMP-ALG/HA* group [injected with vancomycin (50 mg/mL)/BMP-2 (1 μg/mL)-incorporated ALG/HA hydrogel into the osteomyelitis femur]. To induce femur osteomyelitis in rat, 100 μL of bacteria (*S. aureus*) solution [10^4 CFU (colony-forming unit)/mL] was injected into the medullary cavity using 18G needle. The detailed procedures to develop femur osteomyelitis were described in our previous study [39]. At 2 weeks after bacteria inoculation, the infected medullary cavity of femur (femur osteomyelitis) was re-exposed and the defect site was injected with 100 μL of hydrogel (*ALG/HA*, *VAN-ALG/HA,* or *VAN/BMP-ALG/HA*) using 18G needle. The soft tissue incisions were sutured using a 5–0 nylon suture (Ethicon, Cincinnati, OH, USA). At predesignated periods (3 and 6 weeks), the animals were euthanized by an overdose of CO_2 gas, and each femur was harvested for radiographic, biomechanical and microbiological examinations. The femurs were scanned with a micro-computed tomography (μ-CT; Skyscan 1176, Skyscan N.V., Kontich, Antwerp, Belgium) to estimate the severity of osteomyelitis. The biomechanical properties of the harvested femurs were determined using a universal testing machine (UTM, AG-5000G; Shimadzu, Kyoto, Japan) to compare the strength among normal bone, infected bone (osteomyelitis), and hydrogel-treated bones. Femur specimens were prepared using a modified method designed by Skripitz and Aspenberg [41]. Both ends of femur sample were embedded using PMMA. After polymerization of the PMMA, the specimen was fixed at the UTM (50 kgf load cell) and the specimen was pulled at a crosshead speed of 60 mm/min. Then the ultimate load of the specimen was measured.

At 3 and 6 weeks after surgery (hydrogel injection), the infective femurs were explanted under aseptic conditions and a microbiological examination was conducted immediately. The harvested femurs were homogenized in a dismembranator (Braun, Melsungen, Hessen, Germany), vortexed in sterile 0.9% saline solution (10 mL) for 3 min and centrifuged (3000 rpm) for 5 min. The supernatant (50 μL) of each sample was plated on nutrient agar. After 1 day incubation at 36 °C, the number of bacteria in each femur specimen was quantified by colony counting. To investigate changes in blood composition associated with the infection, blood samples (3 mL) of each group were harvested by cardiac puncture [42] at 3 and 6 weeks after injection of hydrogel. The number of white blood cells (WBCs) and the hemoglobin level were determined using an ADVIA 2120 hematology analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

2.7 Statistical analysis

The data obtained from each group were expressed as mean ± standard deviation. Statistical analyses were conducted using IBM SPSS software (IBM, Chicago, Il, USA). Data were evaluated using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Comparisons with $p < 0.05$ were regarded as statistically significant.

3 Results and discussion

3.1 Characterization of vancomycin- and BMP-2-incorporated ALG/HA hydrogel

The *in situ* gelling ALG/HA hydrogels (*ALG/HA*, *VAN-ALG/HA, BMP-ALG/HA,* and *VAN/BMP-ALG/HA)* were prepared simply by mixing each ALG/Na_2HPO_4-containing solution and $CaSO_4$-contining solution to control gelation rate. The gelation time of ALG/HA hydrogels was approximately 4 min, which is sufficient time for handling and injection into osteomyelitis lesion (medullry cavity). In our previous studies [30, 43], it was demonstrated that the gelation rate of the ALG hydrogel can be regulated by the ratio of $CaSO_4$ (Ca^{2+} ion source for crosslinking of ALG) and Na_2HPO_4 (Na^+ ion source for retardation of ALG crosslinking via Ca^{2+} ion) and that the gelation rate is not notably influenced by the addition of hydrophilic polymer (e.g., HA) or protein (e.g., BMP-2). It is well-established that hydrogels with sol–gel transition behavior (i.e., *in situ* gelling hydrogel) can be a suitable delivery system for bioactive molecules (e.g., drugs, growth factors, cytokines, and hormones) because of their easy encapsulation of bioactive molecules, prolonged preservation of activity of bioactive molecules, and simple application (injection) into defect sites without invasive surgery [44–46]. Therefore, we expected that the ALG/HA hydrogel would be an appropriate matrix for delivery of vancomycin and BMP-2 to treat osteomyelitis.

3.2 Release behavior of vancomycin and BMP-2

The vancomycin and BMP-2 release behaviors from the *VAN/BMP-ALG/HA* hydrogels (30–70 mg/mL vancomycin and 1 μg/mL BMP-2 loading) were investigated for up to 6 weeks. The vancomycin release pattern from the

hydrogels with different vancomycin concentrations showed that the vancomycin was continuously released for up to 6 weeks (Fig. 2A), even though vancomycin is a water-soluble and low molecular weight (1486 g/mol) drug. The release tendency among the hydrogels was not significantly different, but the amounts they releases was proportional to the loading amount, which was attributed the charge–charge interaction between negatively charged ALG and positively charged vancomycin as mentioned earlier, and slow dissociation of the drug from the ALG in the physiological environment (e.g., PBS, body fluid, and blood) [34]. The continuous and prolonged release of vancomycin, which is commonly used as an antibiotic to treat MRSA-infected lesions [35, 36], can meet the essential requirement of being a local delivery system for the treatment of osteomyelitis.

To confirm the charge–charge interaction between ALG and vancomycin, the vancomycin (50 mg/mL) was incorporated in negatively charged ALG and neutrally charged Pluronic F127 solutions, and the vancomycin release behavior from the solutions was compared (Fig. 2B). While almost all vancomycin in Pluronic F127 solution was rapidly released within 10 h, the release of vancomycin in ALG solution was significantly delayed (~ 27% after 36 h) compared with the Pluronic F127 solution, suggesting that, as expected, the positively charged vancomycin can form charge–charge interactions between oppositely charged ALG chains which can interfere in direct liberation of the vancomycin.

The BMP-2 release pattern from the *VAN/BMP-ALG/HA* hydrogel (1 μg/mL BMP-2 and 50 mg/mL vancomycin loading) was shown in Fig. 2C. The hydrogel showed relatively fast release of BMP-2 at the first day, but after that time there was sustained release of BMP-2 for 6 weeks. This result can be understood as fast desorption of BMP-2 located (adsorbed) at the surface region of the hydrogel (initial burst), and then retarded liberation of BMP-2 through the pore network in the hydrogel (slow release) [47]. The release pattern of the BMP-2 was very similar to that of the *BMP-ALG/HA* hydrogel without vancomycin (data not shown), indicating the vancomycin incorporated in the ALG/HA hydrogel does not lead to any significant interference on the release of BMP-2. As well-established by other literature [48, 49], the sustained release of BMP-2

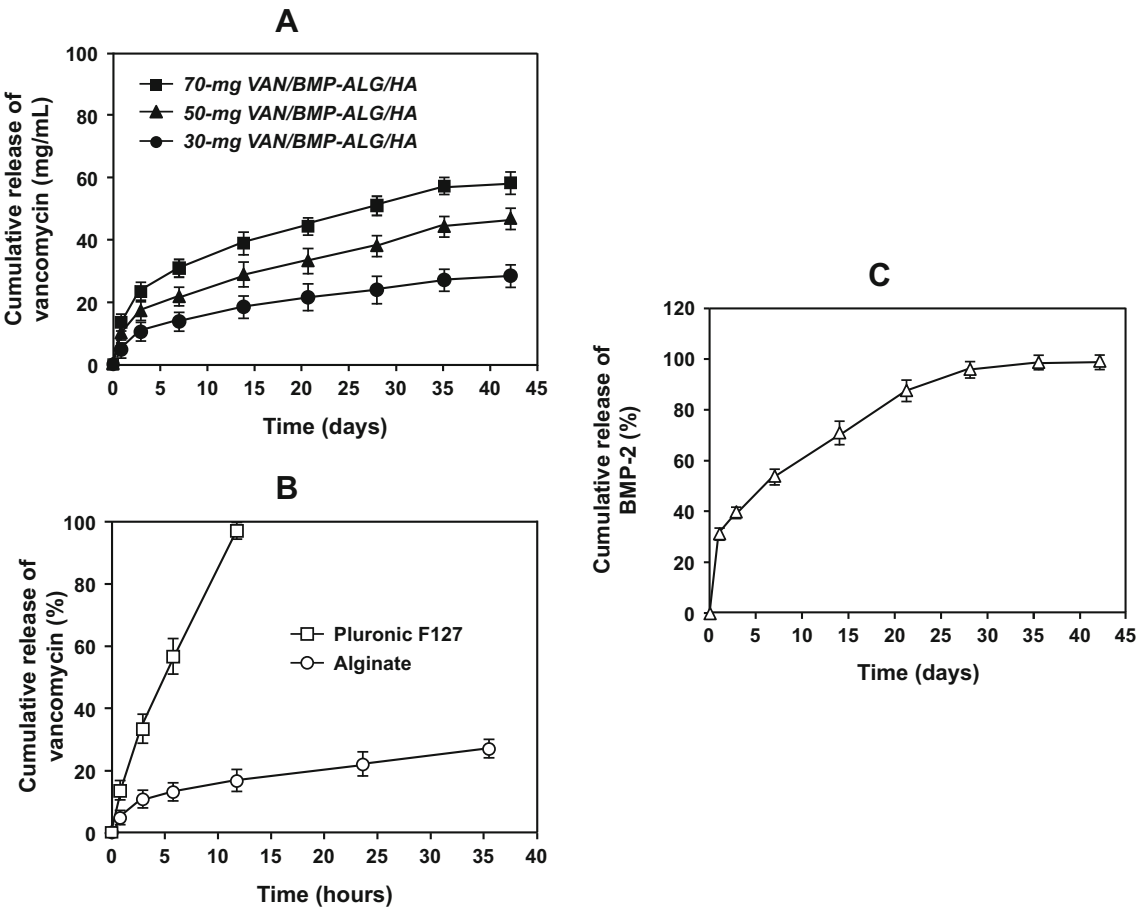

Fig. 2 Cumulative release behaviors of **A** vancomycin from *VAN/BMP-ALG/HA*, **B** vancomycin from ALG and Pluronic F127 solutions, and **C** BMP-2 from *VAN/BMP-ALG/HA* (n = 3)

can lead to prolonged activity of BMP-2 and thus allow effective osteogenic differentiation of stem cells and bone reconstruction.

3.3 Cytotoxicity of hydrogels

The results for shows cytotoxicity of vancomycin released from the *VAN/BMP-ALG/HA* hydrogels with different vancomycin concentrations show that the cell viability of the hydrogels was slightly decreased with increasing concentration of vancomycin (Fig. 3); however, there was no siginficant difference in the hydrogels between the vancomycin concentrations up to 50 mg/mL, indicating that the concentration of vancomycin up to 50 mg/mL incorporated in the *VAN/BMP-ALG/HA* hydrogel does not lead to notable cytotoxicity. The release amounts of vancomycin at first day of 30-mg and 50-mg *VAN/BMP-ALG/HA* were 6.7 ± 1.1 mg/mL and 10.0 ± 1.4 mg/mL, respectively (Fig. 2A). It was reported that a concentration of vancomycin over 5 mg/mL leads to notable toxicity in corneal endothelial cells [50]. Although it is a comparison between different cell types, the improved cell viability of the *VAN/BMP-ALG/HA* hydrogel on vancomycin concentration may be explained by the cytoprotective effect of the BMP-2 [51] simultaneously released with vancomycin from the hydrogel.

3.4 *In vitro* anti-bacterial activity of hydrogels

In vitro anti-bacterial activity of the *VAN/BMP-ALG/HA* hydrogels (30–70 mg/mL vancomycin and 1 μg/mL BMP-2 loading) was compared with PBS, *ALG/HA* hydrogel (without vancomycin and BMP-2), and free vancomycin [30–70 mg/mL in PBS (Fig. 4)]. As expected, the PBS and *ALG/HA* group without antibiotic did not show any anti-bacterial activity. This also indicates that the extractant from the *ALG/HA* hydrogel at each time point does not lead to any positive effect on anti-bacterial activity. In the case of free vancomycin groups, the anti-bacterial activity was significant at first day, but the activity had totally disappeared at second day because of rapid release (dissolving) of vancomycin within 1 day, regardless of vancomycin concentration. On the other hand, the *VAN/BMP-ALG/HA* hydrogel groups showed prolonged anti-bacterial activity for 6 weeks, which is the period required for effective treatment of osteomyelitis [39]. The *VAN/BMP-ALG/HA* hydrogels with greater concentration of vancomycin showed larger ZOI than those with lower concentrations, regardless of period, indicating better anti-bacterial activity. The ZOI of *VAN/BMP-ALG/HA* groups gradually decreased with time, probably owing to the gradual reduction of vancomycin release amount with time from the hydrogels (Fig. 2A). From the *in vitro* findings of the cytotoxicity and anti-bacterial activity tests, the *VAN/BMP-ALG/HA* hydrogel with vancomycin concentration of 50 mg/mL was selected as a candidate to treat osteomyelitis for animal study.

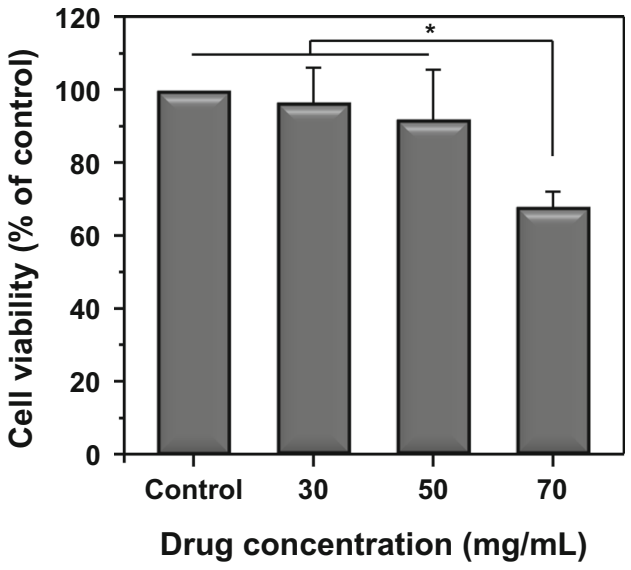

Fig. 3 Cell (MC3T3-E1) viability as a function of vancomycin concentration in *VAN/BMP-ALG/HA* (n = 3; *$p < 0.05$)

3.5 *In vivo* anti-bacterial activity and bone regeneration of hydrogels

To investigate the anti-bacterial activity (by vancomycin) and bone regeneration (by BMP-2) of the *VAN/BMP-ALG/HA* hydrogels, SD rats (femur osteomyelitis model) were used. At 3 and 6 weeks after injection of the ALG/HA hydrogels (*ALG/HA, VAN-ALG/HA,* and *VAN/BMP-ALG/HA*) into osteomyelitis femur, the severity of osteomyelitis was evaluated by radiographic (Fig. 5), biomechanical (Fig. 6), and microbiological (Fig. 7) examinations. All ALG/HA-based mixture solutions were easily injected into the medullary cavity using 18G needle syringe and subsequently transformed into hydrogels which allow stable maintenance of the injected ALG/HA-based materials in the cavity without leakage. In the osteomyelitis group, bone destruction (sponge-like bone) was observed in the femur bone at 3 weeks, and the severity of damage gradually worsen over time (Fig. 5). This indicates that *S. aureus* inoculated in the femoral medullary cavity was effective for development of osteomyelitis [52]. The *ALG/HA* group without vancomycin and BMP-2 showed a similar tendency with the osteomyelitis group, indicating that the *ALG/HA* hydrogel does not inhibit the proliferation of *S. aureus* in the medullary cavity. On the other hand, the severity of osteomyelitis in the hydrogel groups containing vancomycin (*VAN-ALG/HA* and *VAN/BMP-ALG/HA*)

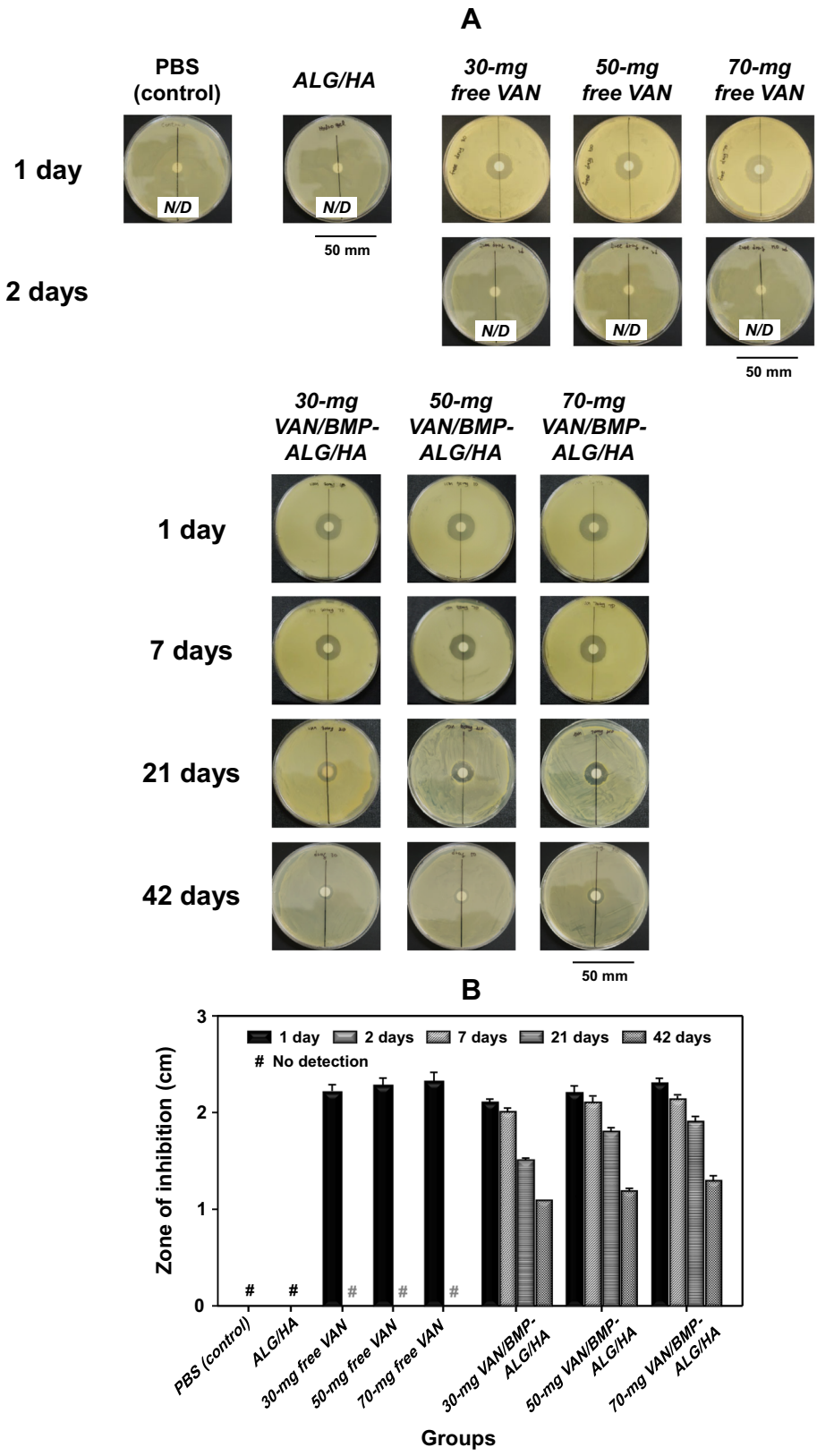

Fig. 4 Anti-bacterial activity (ZOI of *S. aureus*) of PBS, *ALG/HA*, *free VAN* (vancomycin concentrations, 30–70 mg/mL in PBS), and *VAN/BMP-ALG/HA* (vancomycin concentration, 30–70 mg/mL in hydrogel) groups as a function of time. **A** Photographs showing ZOI (*N/D*, no detection) and **B** their calculated result (n = 3)

decreased (less bone destruction) compared with the osteomyelitis and *ALG/HA* groups, probably because of continuous release of vancomycin from the hydrogel for 6 weeks (Fig. 2) and thus effective suppression of the *S. aureus* proliferation in the infected site. Moreover, the femur injected with the *VAN/BMP-ALG/HA* hydrogel

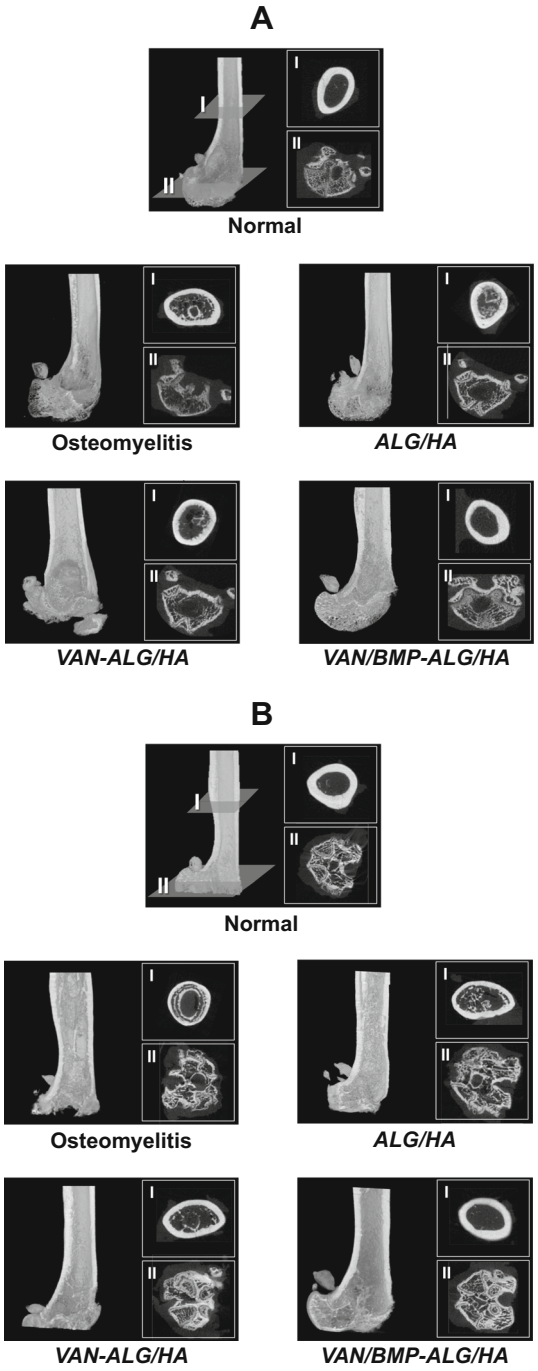

Fig. 5 Micro-CT images of normal femur, osteomyelitis femur, and hydrogel (osteomyelitis femur treated by *ALG/HA*, *VAN-ALG/HA*, and *VAN/BMP-ALG/HA*) groups at **A** 3 and **B** 6 weeks after surgery

showed denser bone reconstruction than the other groups, regardless of period.

The biomechanical strength of all ALG/HA hydrogel groups was lower than that of the normal group because of the bone destruction during the osteomyelitis development stage for 3 weeks after bacteria inoculation (Fig. 6). In general osteomyelitis, the compact (cortical) bone is

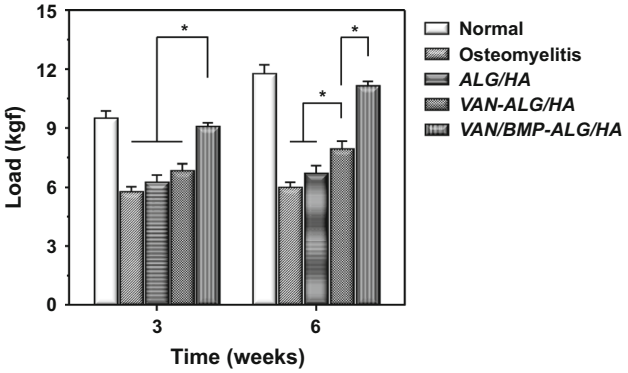

Fig. 6 Biomechanical strength of normal femur, osteomyelitis femur, and hydrogel (osteomyelitis femur treated by *ALG/HA, VAN-ALG/HA*, and *VAN/BMP-ALG/HA*) groups at 3 and 6 weeks after surgery (n = 3; $*p < 0.05$)

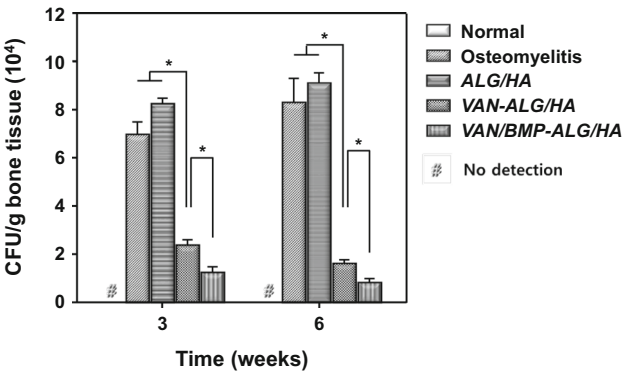

Fig. 7 CFU of *S. aureus* in normal femur, osteomyelitis femur, and hydrogel (osteomyelitis femur treated by *ALG/HA, VAN-ALG/HA*, and *VAN/BMP-ALG/HA*) groups at 3 and 6 weeks after surgery (n = 3; $*p < 0.05$)

transformed into a sponge-like structure [52]; thus the biomechanical properties decline. The severity of osteomyelitis based on biomechanical strength in the *ALG/HA* group (without vancomycin and BMP-2) was not notably different from that of the osteomyelitis group (decreased biomechanical strength due to bone loss) [3, 7, 8]. The *VAN-ALG/HA* group showed higher biomechanical strength than the osteomyelitis and *ALG/HA* groups, probably because of suppression of *S. aureus* growth by the sustained release of vancomycin and inhibition of proceeding bone loss. The biomechanical strength in the *VAN-ALG/HA* group gradually increased with time, indicating self-healing of the defect bone. The *VAN/BMP-ALG/HA* group, which supplies both vancomycin and BMP-2 for 6 weeks, showed significantly increased biomechanical strength compared with the *VAN-ALG/HA* group and the strength was not notably different from that of normal bone, even without the use of additional bone grafts. These findings can be explained by the combined effect of the anti-bacterial activity with continuously

released antibiotic and the induction of bone regeneration by sustained supply of BMP-2. Nguyen et al. [53] demonstrated that vancomycin as an antibiotic and BMP-2 as a growth factor preserve their own functionality when both bioactive molecules are co-administered in a co-culture system of *S. aureus* and bone marrow stromal cells. Moreover, their co-administration can achieve more effective suppression of *S. aureus* proliferation compared with a vancomycin alone group. The results of co-administration were explained as being because the rapid proliferation of bone marrow stromal cells by BMP-2 outpaces the infection and growth of bacteria.

To evaluate infection persistence in the medullary cavity infected by *S. aureus* and treated by ALG/HA hydrogels, all infective femurs were explanted and microbiological examination performed on each. As expected, no positive effect suppresing the proliferation of bacteria was found in the osteomyelitis and *ALG/HA* groups, regardless of period (Fig. 7). However, the number of bacteria (colony) was effectively inhibited in the vancomycin-incorporated groups (*VAN-ALG/HA* and *VAN/BMP-ALG/HA*), probably because of the continuous supply of bioactive antibiotic with therapeutic concentration for a sufficient period of time at the infected femur, which can not be found in parenteral or oral delivery systems [12–14]. The enhanced anti-bacterial activity of *VAN/BMP-ALG/HA* may be understood by the proliferation/differentiation of bone cells by BMP-2 outpacing that of bacteria [53].

To estimate the changes in blood composition related to inflammatory diseases (i.e., infection) at 3 and 6 weeks after the injection of the ALG/HA hydrogels, the number of WBCs and the hemoglobin level were measured using whole blood obtained from each animal group (Fig. 8). The number of WBCs in the osteomyelitis and *ALG/HA* groups increased notably compared with the normal group, regardless of period, suggesting the development of osteomyelitis (Fig. 8A). On the other hand, the number of WBCs in the vancomycin-incorporated hydrogel groups (*VAN-ALG/HA* and *VAN/BMP-ALG/HA*) was not notably different from that in the normal group, indicating that the continuously released vancomycin from the ALG/HA hydrogel suppressed effectively the proliferation of the *S. aureus* inoculated in femur and thus stablized the number of WBC associated with inflammation. At 3 and 6 weeks after injection of the ALG/HA hydrogels, the differences of hemoglobin level among the group were not significant (Fig. 8B). These observations are consistent with the results of drug release and the radiographic, biomechanical, and microbiological examinations, which show that the vancomycin-incorportated hydrogel groups allow continuous release of vancomycin, and the drug inhibits effectively the growth of bacteria inoculated in femur, which leads to bone loss. From the *in vitro* and *in vivo* findings, we believe

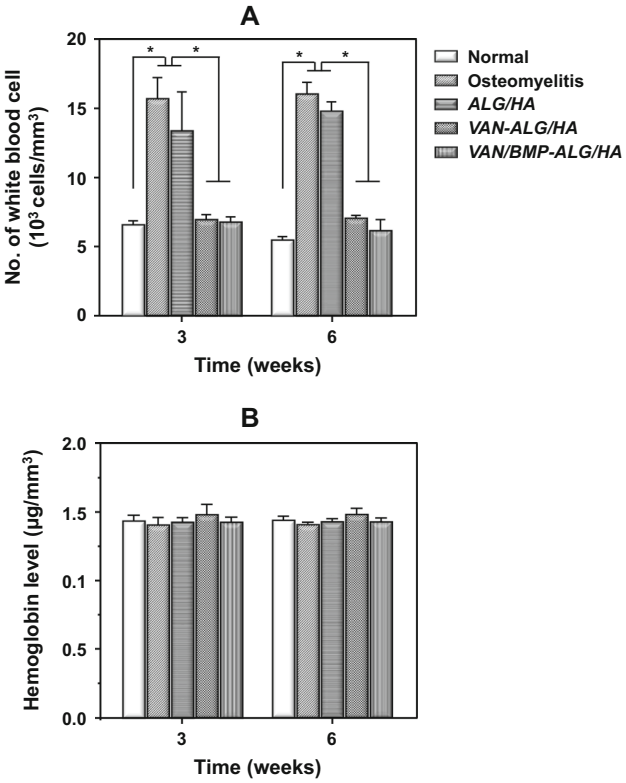

Fig. 8 **A** Number of white blood cells and **B** hemoglobin level in the normal femur, osteomyelitis femur, and hydrogel (osteomyelitis femur treated by *ALG/HA, VAN-ALG/HA*, and *VAN/BMP-ALG/HA*) groups at 3 and 6 weeks after surgery (n = 3; $*p < 0.05$)

that the *VAN/BMP-ALG/HA* hydrogel may be a feasible one-step therapeutic system to reduce the burden of patients and clinicians in conventional two-step treatment (i.e., infection control and subsequent bone reconstruction [9]) for osteomyelitis. Moreover, *ALG/HA* hydrogel can be a delivery system of negatively charged and susceptive antibiotics for osteomyelitis (e.g., minocycline, fluoroquinoline, and clindamycin [54, 55]) as well as of growth factors associated with new bone formation [e.g., BMP-7, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB), transforming growth factor-β (TGF-β), and basic fibroblast growth factor (bFGF) [56] for more systematic investigation into the treatment of osteomyelitis.

4 Conclusions

An *in situ* gelling ALG/HA hydrogel containing antibiotic (vancomycin) and growth factor (BMP-2) was prepared. The gelation time of the ALG/HA hydrogel was controlled by the ratio of $CaSO_4$ and Na_2HPO_4 to be approximately 4 min, which is sufficient time for handling and injection into the osteomyelitis lesion (medullary cavity) selected for

this study. The vancomycin and BMP-2 were continuously released from the hydrogel for up to 6 weeks. The *VAN/BMP-ALG/HA* hydrogel (containing 50 mg/mL vancomycin and 1 μg/mL BMP-2) was effective in *in vitro* anti-bacterial activity without significant cytotoxicity for 6 weeks, which is the required period for effective treatment of osteomyelitis. From an animal study, it was demonstrated that the ALG/HA hydrogel is effective in suppressing *S. aureus* proliferation at the osteomyelitis lesion and enhancing bone regeneration without additional bone grafts. Thus, the *in situ* gelling ALG/HA hydrogel containing vancomycin and BMP-2 may be a feasible therapeutic tool for osteomyelitis and reduce the burden of patients and clinicians caused by two-step surgical treatment (1st infection control and 2nd bone regeneration).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical statement All animal studies were approved from the Animal Care Committee of Hannam University of Korea (HNU-IACUC 2016-14).

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