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In vitro evaluation of an injectable chitosan gel for sustained local delivery of BMP-2 for osteoblastic differentiation

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Abstract: We investigated the effect of sustained release of bone morphogenetic protein-2 (BMP-2) from an injectable chitosan gel on osteoblastic differentiation *in vitro*. We first characterized the release profile of BMP-2 from the gels, and then examined the cellular responses of preosteoblast mouse stromal cells (W-20-17) and human embryonic palatal mesenchymal (HEPM) cells to BMP-2. The release profiles of different concentrations of BMP-2 exhibited sustained releases (41% for 2 ng/mL and 48% for 20 ng/mL, respectively) from the chitosan gels over a three-week period. Both cell types cultured in the chitosan gels were viable and significantly proliferated for 3 days ($p < 0.05$). Chitosan gels loaded with BMP-2 enhanced ALP activity of W-20-17 by 3.6-fold, and increased calcium mineral deposition of HEPM by 2.8-fold at 14 days of incubation, compared to control groups initially containing the same amount of BMP-2. In addition, schitosan gels loaded with BMP-2 exhibited significantly greater osteocalcin synthesis of W-20-17 at seven days, and of HEPM at both 7 and 14 days compared with the control groups ($p<0.05$). This study suggests that the enhanced effects of BMP-2 released from chitosan gels on cell differentiation and mineralization are species and cell type dependent. J Biomed Mater Res Part B: Appl Biomater 99B: 380–390, 2011.

Key Words: injectable gel, chitosan, β-glycerophosphate (β-GP), BMP-2, biocompatibility, release profile, osteoblastic differentiation, W-20-17, HEPM

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INTRODUCTION

In orthopedic applications, osteoinductive growth factors have been widely studied to regulate cellular activities such as cell growth, proliferation, and differentiation for bone fracture healing and bone formation.[1–3] Bone morphogenetic protein-2 (BMP-2) is one of the most potent growth factors approved by the FDA for complete bone morphogenesis (e.g., bone remodeling and bone formation).[1,4–6] BMP-2 has been shown to enhance bone formation by inducing osteoblastic differentiation in mesenchymal stem cells (MSCs).[7,8] To treat a bone defect in clinical applications, a large dose of BMP-2 is generally administered by single or multiple injections.[5,6] However, these treatments are inefficient because BMP-2 rapidly loses physical stability and bioactivity *in vivo*.[9–11] In conventional therapies, a common problem is the initial burst release, inducing a rapid loss of the efficacy of the growth factor and subjecting the body to harmful side effects. In order to get optimal results, BMP-2 needs to be combined with an appropriate carrier to extend its residence time.[10,11] In this regard, implantable local delivery systems have gained much attention for their prospective capacity to prolong therapeutic span while minimizing side effects.

A local delivery system, in general, is a device that can carry and discharge therapeutic drugs, growth factors, and cells to specific sites of the human body for a desired time period.[12,13] Recent studies have shown the significance of combining biomaterials with BMP-2 in the field of bone regeneration.[9] BMP-2 treatment using bone-forming materials had a positive effect on the repair of bone defects, promoting alkaline phosphatase (ALP) activity and increasing mineralized matrix formation *in vitro* and *in vivo*.[14–17] A variety of

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tissue-engineered materials have been used for the controlled steady release of BMP-2 to treat bone defects by optimizing therapeutic efficacy. Encapsulation of BMP-2 in such material structures protects its bioactivity for a long time period and stimulates desired cellular responses.[9,10,15,16]

In recent years, thermo-sensitive injectable systems have gained special attention due to their ability to deliver therapeutic agents, molecules or cells.[18–23] Injectable delivery systems have several advantages over implantable drug delivery systems.[18,24–26] An injectable gel formulation is liquid at room temperature, and becomes a gel at body temperature. Using such a system, a variety of therapeutics (such as hydrophobic drugs, various types of growth factors, or even cells) can be directly administered at the targeted site by a simple injection without surgical intervention.[20,21,23–26] This delivery method can reduce costs and surgical complications. Most recently, a chitosan based injectable gel combined with β-glycerophosphate (β-GP) has shown notable potential as a nonsurgical delivery system due to its ability for controlled release and high loading capacity.[27–30] In particular, chitosan has been considered a promising biodegradable polymer for controlled drug release with respect to safety and efficacy of drug therapy.[22,23,31] Therapeutic agents are chemically attached or dispersed into the chitosan matrix and are released via slow and controllable diffusion or biodegradation.[32–34] In addition, it has been reported that the chitosan polymer itself can stimulate the activity of growth factors.[11] In the other hand, β-GP has been known as a source of organic phosphate for mineralization and used for osteogenic differentiation in combination of other *in vitro* inducers such as dexamethasone, ascorbic acid, and vitamin D_3.[35–38] In this study, a chitosan polymer was used as a drug carrier while a β-GP was employed to induce a thermo-sensitive sol–gel transition.

In our previous study,[30] we found that an injectable chitosan/β-GP gel underwent thermal gelation at body temperature. Dialysis of an acidic chitosan solution reduced the amount of β-GP needed for gelation, thereby significantly improving cell viability *in vitro*. In the present study we characterized an injectable thermo-sensitive chitosan gel's capacity to effectively deliver bone morphogenetic protein-2 (BMP-2) over a long term period *in vitro. We also* evaluated the effect of BMP-2 released from the gels or added into medium on the behaviors of two cell lines, preosteoblast mouse bone marrow stromal cells (W-20-17) and human embryonic palatal mesenchymal cells (HEPM). We hypothesized that the injectable chitosan gel would effectively deliver BMP-2 in a sustained release fashion to stimulate differentiation and mineralization of bone cells.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Sigma Aldrich in high molecular weight ($\geq$ 310 KDa) with an ~75% or greater degree of deacetylation. Disodium β-GP (glycerol 2-phosphate disodium salt hydrate; cell culture grade) was purchased from Sigma Aldrich. Human bone morphogenetic protein-2 (BMP-2) was obtained from PeproTech, Inc. (Rocky Hill, NJ). All other chemicals were reagent grade and used as received. Fetal bovine serum (FBS), Trypsin-EDTA, L-Glutamine, Antibiotic-Antimycotic, Phosphate buffered saline (PBS), and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco™. The preosteoblast mouse bone marrow stromal cells (W-20-17) and human embryonic palatal mesenchymal cells (HEPM) were cultured as per American Type Culture Collection (ATCC) instructions.

Preparation of an injectable chitosan gel

A 1.5% (w/v) chitosan solution was prepared by stirring powdered chitosan in 0.75% (v/v) aqueous acetic acid at room temperature overnight. The insoluble particles in the chitosan solution were removed by filtration. A 50% (w/v) β-GP solution was prepared in distilled water and sterilized using PES syringe filters with 0.22-μm pore size (MillexTM, MA) and stored at 4°C. Totally, 50 mL of chitosan solution was dialyzed at room temperature against 1 L of distilled water for seven days with daily changes of water (1 L) in an 8 kDa cutoff dialysis membrane to reduce the acetic acid content. The final pH value of the chitosan solution was 6.3. The dialyzed chitosan solution was autoclaved at 121°C for 20 min, cooled down to room temperature, and stored at 4°C. Sterilized, ice-cold β-GP solution (2.31*M*) was added drop by drop to the chitosan solution under stirring conditions in an ice bath. The final pH value of the chitosan gel formulation (dialyzed chitosan/β-GP solution) was around 7.2.

In vitro BMP-2 release study from the chitosan gel

The *in vitro* release profile of BMP-2 from the chitosan gel was investigated for a three-week period. BMP-2 is a homodimer linked by a single disulfide bond with an apparent molecular weight of 26 kDa. Its residues are almost electronically neutral at pH 7.2,[39,40] which is the pH value of chitosan gel formulation.

Two different concentrations of BMP-2 were loaded into the dialyzed chitosan solutions and agitated at room temperature and cooled down to 5°C on ice. Cold 88 mM of β-GP solution was added into each mixture to complete the gel forming solution. The chitosan gels loaded with BMP-2 were placed in containers containing 2.5 mL of PBS (pH 7.4) at 37°C, and the initial concentrations of BMP-2 in the release study system were 2 and 20 ng/mL. At designated time points, 350 μL aliquots of the release medium were sampled and the same amount of fresh PBS (pH 7.4) was added into each container. In the collected fractions, the cumulative amount and percentage of BMP-2 released from the chitosan gel were determined as a function of time using a BMP-2 ELISA kit (R&D systems, MN). Totally, 50 μL of the obtained supernatant was pipetted into a 96-well BMP-2 microplate coated with a mouse monoclonal antibody. After washing each well with wash buffer provided by the ELISA kit for a total of four washes, 200 μL of BMP-2 conjugate was added to each well and the samples were incubated at room temperature for 2 h. After repeating the washing step, each well was filled with 200 μL of BMP-2 substrate and incubated at room temperature for 30 min in the dark. Finally, 50 μL of stop solution was added into each well. The optical density of each well was determined

using a microplate reader at 450 nm with a correction setting of 540 nm.

Cell culture

The preosteoblast mouse bone marrow stromal cells (W-20-17) and human embryonic palatal mesenchymal cells (HEPM) were grown and maintained in DMEM media with 10% FBS, 1% antibiotic/antimycotic mixture, 5 mL of L-glutamine (200 m*M*), and sodium pyruvate. The cells were cultured in an incubator supplied with 5% CO_2 at 37°C. The culture medium was changed every three days.

Cytotoxicity of chitosan gels *in vitro*

The cytotoxicity of the chitosan gels on W-20-17and HEPM was quantitatively examined by a MTS assay via an indirect cell culture, in which cells were not in direct contact with gels that were contained in inserts. In addition, viable cells directly encapsulated into the chitosan gels were observed qualitatively by a microscope (Nikon ECLIPSE TE-2000-U) using the live/dead viability assay.

Indirect culture via BD BioCoat™ Control Cell Culture inserts (12-well formats) was used to evaluate the cytotoxicity of the gels through cell culture media. The insert contains a 0.45 μm pore size PET membrane with a thin layer of basement membrane matrix which prevents cells from migrating through the membrane and allows only the culture medium through the PET membrane. A dialyzed chitosan solution (pH 6.3) was mixed with 88 m*M* of β-GP solution and its final pH value was around 7.23. Totally, 1 mL of each chitosan gel forming solution was placed into the bottom of well plates and incubated at 37°C for 1 h prior to cell culture. A total of 1.7 mL of the cell culture medium was added into each well of the 12-well plates with inserts, which were allowed to hydrate for 2 h at 37°C. The cells were then seeded at a density of 35,000 cells/well in the upper chamber with 0.3 mL of culture medium. After incubation for one, two, and three days, the number of viable cells was determined quantitatively using a Cell Titer 96AQueous One Solution assay according to the manufacturer's instructions.

Photomicrographs of cells were also documented qualitatively during cell culture using a microscope and processed using MetaVue software. Cells were directly seeded on the chitosan gels in 24-well plates at a density of 60,000 cells/well and incubated for three days, after which the cells were stained with the live/dead viability assay (Invitrogen detection technologies) and then cell morphology was observed. Viable cells were stained with calcein AM and appeared green, and dead cells were stained with ethidium homodimer-1and appeared red.

Cell responses to BMP-2 released from the gels: preparation of cell lysates

Indirect culture via BD BioCoat™ Control Cell Culture inserts was used as a model system to evaluate the cell responses to the BMP-2 released from the injectable chitosan gels. Cells were seeded in the bottom of 12-well plates at a density of 15,000 cells/well. In the experimental group, the chitosan gels loaded with BMP-2 were placed in the upper chamber of inserts. The cells were cultured in DMEM supplemented with 10% FBS. In order to enhance cell response, 50 ng/mL of BMP-2 was used in each group. The culture medium was completely changed weekly. Two control groups did not contain the chitosan gels. In the negative control group, cells were incubated in DMEM without BMP-2. In the positive control group, the cells were cultured in DMEM containing 50 ng/mL of BMP-2 initially, but it did not get further replenishment of BMP-2 after one week. The positive control group and the experimental gel group initially contained the same amount of BMP-2. The positive control group was designated for a burst release effect of BMP-2. And the experimental gel group was designated for a sustainable release effect of BMP-2. At designated time points (7, 14, and 21 days), the medium was removed from the cell culture. The cell layers were washed twice with PBS (pH 7.4) and then lysed with 1 mL of 0.2% Triton X-100 by three freeze-thaw cycles, which consisted of freezing at −80°C for 30 min immediately followed by thawing at 37°C for 15 min. Cell behaviors of W-20-17 and HEPM were determined by ALP activity, double stranded DNA, total protein, and osteocalcin production. Calcium mineralization of cells was characterized by Alizarin Red S staining imaging and quantification.

ALP activity

The cell lysates were assayed for the presence of ALP, an important marker for determining osteoblast phenotype. Totally, 50 μL aliquots of the cell lysates were sampled and added to 50 μL of working reagent in a 96-well assay plate. The working regent contains equal parts (1:1:1) of 1.5*M* 2-amino-2-methyl-1-propanol (Sigma), 20 m*M* p-nitrophenyl phosphate (Sigma), and 1 m*M* magnesium chloride. The samples then were incubated for 1 h at 37°C. After incubation, the reaction was stopped with 100 μL of 1*N* sodium hydroxide on ice. ALP activity was determined from the absorbance using a standard curve prepared from p-nitrophenol stock standard (Sigma). The absorbance was measured at 405 nm using a microplate reader (Bio-Rad model 680). The ALP activity of cells was then calculated by normalizing to double stranded DNA (dsDNA). ALP activity was expressed as nmol/ng.

Double stranded DNA

The cell lysates were examined for double stranded DNA to estimate cell number. Totally, 50 μL aliquots of the cell lysates were added in a 96-well assay plate. Each 50 μL of a 1:200 dilution of picogreen (Quant-iT PicoGreen assay kit, Invitrogen) was added to each well and incubated for 5 min in the dark. The assay plate was read at 485 nm excitation and 530 nm emissions using a BioTek FLx800 plate reader. The double stranded DNA content was calculated using a standard curve made by a provided dsDNA standard sample.

Alizarin Red S staining for calcium mineralization

Calcium mineral content within the cell layers was determined qualitatively and quantitatively by Alizarin Red S

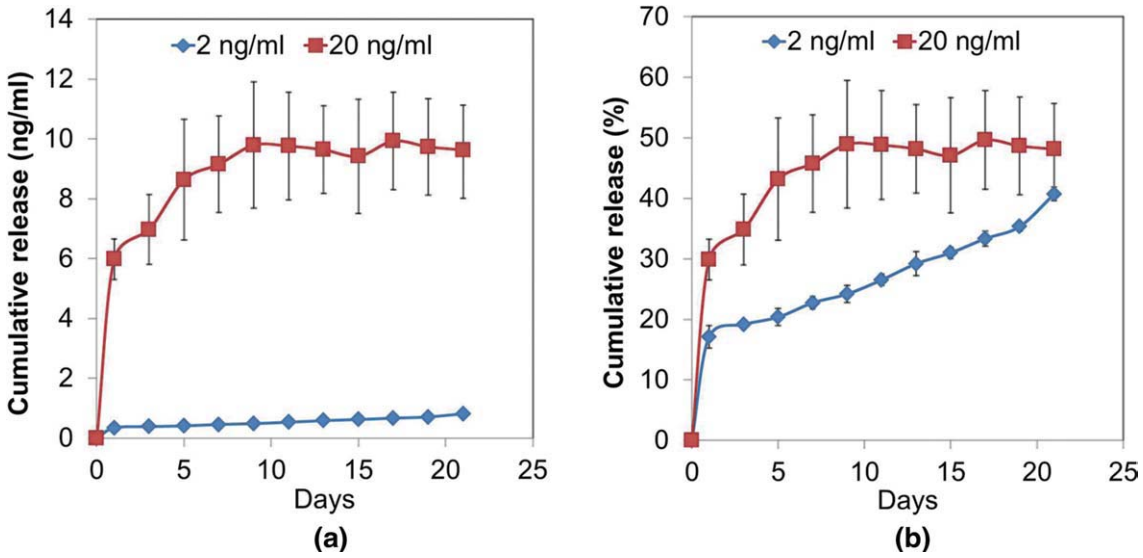

FIGURE 1. *In vitro* cumulative release profiles of BMP-2 from chitosan gels over a three-week period. The cumulative release (a) amount and (b) percentage of BMP-2 from the chitosan gels were determined as a function of time by a BMP-2 immunoassay at 450 nm. Concentration of BMP-2 was 2 or 20 ng/mL. Each value represents the mean ± SD ($n = 4$ per each group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

staining (AR-S). At the end of each time point (7, 14, and 21 days incubation), the cell layers were washed with PBS (pH 7.4) twice and fixed in ice-cold 50% ethanol at 4°C for 30 min. After washing with distilled water, they were completely dried at room temperature and stained by adding 1 mL of 1% Alizarin Red S (10 mg/mL) at room temperature for 45 min. The cell layers were then washed with distilled water five times and dried completely. Stained cell layers were photographed using microscope (Nikon ECLIPSE TE-2000-U). Quantitative calcium mineral contents were measured by a destaining procedure using an extraction solvent containing a mixture of 10% acetic acid and methanol at room temperature for 30 min. Totally, 200 μL aliquots of alizarin red S extracts were then added in a 96-well assay plate. The Alizarin Red S concentrations of the samples and standard was determined at the absorbance at 450 nm and expressed as μg/mL.

Osteocalcin production

The amount of osteocalcin secreted from W-20-17 and HEPM was measured after 7, 14, and 21 days of incubation. The osteocalcin levels are related to bone mineral density and are often used as a marker of the osteoblast function. The osteocalcin was assayed from the media collected from each well at 0–7, 7–14, and 14–21 days.

A mouse osteocalcin ELISA kit (Biomedical Technologies Inc, MA) was used for W-20-17. Briefly, each 25 μL of collected W-20-17 culture media was added in a 96-well plate coated with osteocalcin antibody followed by the addition of 100 μL of osteocalcin antiserum in each well. After incubation for 24 h, all wells were completely washed three times with 300 μL of provided washing buffer (phosphate saline solution). Each well was then incubated with 100 μL of streptavidin-horseradish peroxidase reagent at room temperature for 30 min. After washing, 100 μL of substrate mixture containing equal parts (1:1) of peroxidase substrate TMB (3,3′,5,5′ Tetramethylbenzidine) and hydrogen peroxide solution was added in each well. The plate was then incubated at room temperature in the dark for 15 min. After the addition of 100 μL of stop solution, the absorbance was measured at 450 nm. The osteocalcin production in W-20-17 cells was calculated using a standard curve made by provided osteocalcin standard samples and was expressed as ng/mL.

For HEPM, an intact human osteocalcin ELISA kit (Biomedical Technologies, MA) was used. Each 25 μL of collected HEPM culture media was added in a 96-well plate coated with 1–19 monoclonal antibodies. After the addition of 50 μL of native osteocalcin antiserum in each well, the plate was incubated at room temperature for 1 h followed by additional 50 μL of diluted biotinylated antiserum. After 1-h incubation, all wells were completely washed three times with 300 μL of provided washing buffer and then incubated with 100 μL of streptavidin-horseradish peroxidase reagent at room temperature for 30 min. After washing, 100 μL of substrate mixture containing equal parts (1:1) of peroxidase substrate (3,3′,5,5′ Tetramethylbenzidine) and hydrogen peroxide solution was added in all wells. The plate was incubated at room temperature in the dark for 10 min. After the addition of 100 μL of stop solution, the absorbance was measured at 450 nm. The osteocalcin production in HEPM cells was calculated using a standard curve made by provided osteocalcin standard samples and expressed as ng/mL.

Statistical analysis

All data are presented as mean ± standard deviation. Significant differences in release profile, cytotoxicity of gel, and *in vitro* bioactivity analyses were analyzed by one-way ANOVA test. The differences in groups and experimental time points at any time were considered significant if $p < 0.05$.

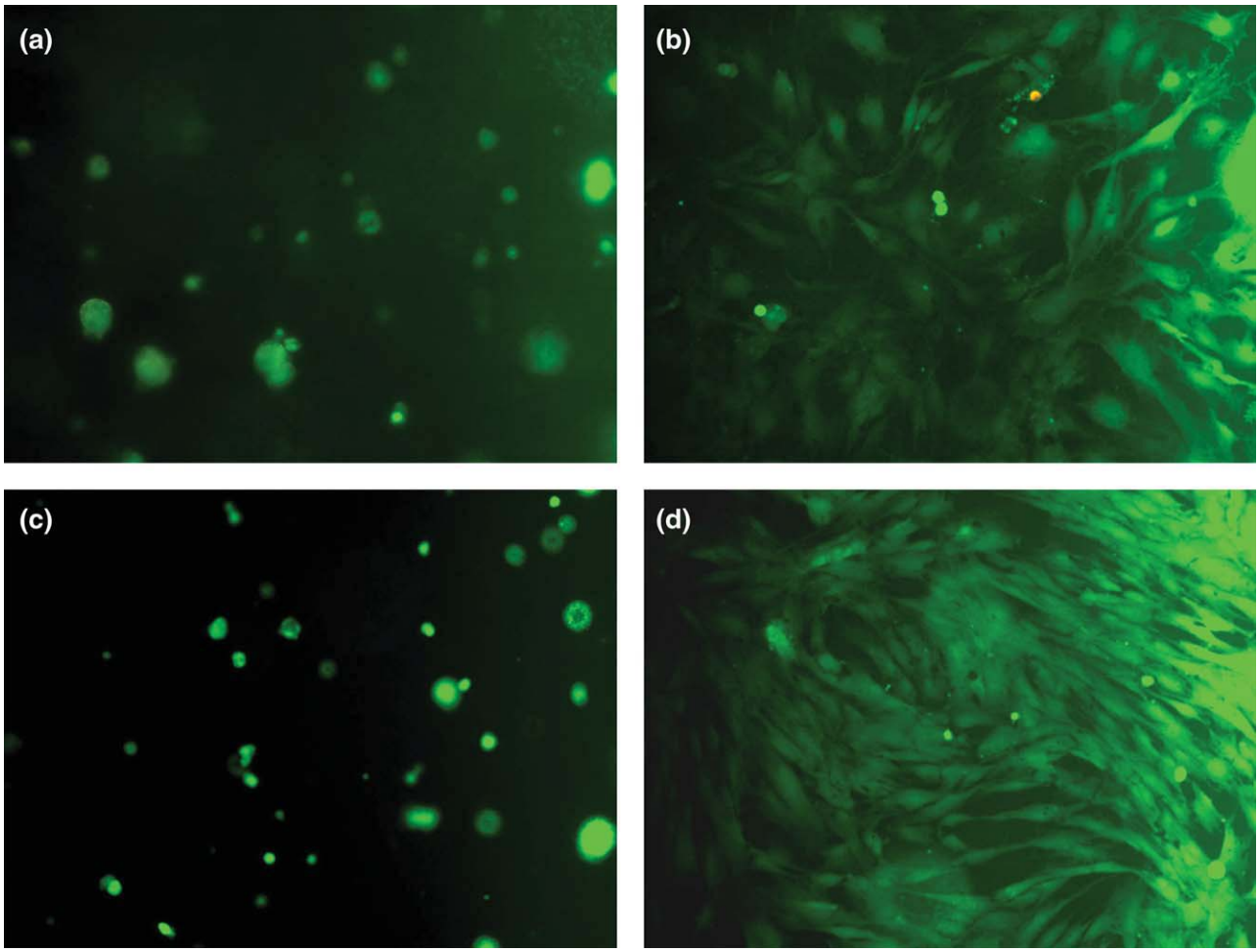

FIGURE 2. Photomicrographs of cell morphology on the chitosan gel via direct culture. (a) W-20-17 cells encapsulated inside gel; (b) W-20-17 cells on the surface of a well plate; (c) HEPM cells encapsulated inside gel; (d) HEPM cells on the surface of a well plate. The cells were cultured on the chitosan gels in 24-well plates at a density of 60,000 cells per well for three days. Viable cells were stained with calcein AM. (MAG = $\times$100).

RESULTS

In vitro BMP-2 release study

The *in vitro* release behaviors of BMP-2 from the chitosan gels were interpreted by the cumulative amount and percentage of the BMP-2 as a function of time. Figure 1(a,b) show the cumulative release of BMP-2 from the chitosan gels. The chitosan gels containing a higher concentration (20 ng/mL) of BMP-2 released significantly greater amounts compared with gels containing a lower concentration (2 ng/ml) [Figure 1(a)] for three weeks. In addition, the chitosan gels containing a higher concentration of BMP-2 (20 ng/mL) exhibited a significantly ($p < 0.05$) higher release percentage by $\sim$1.7-fold at day 1, and 2.2-fold at day 5 compared with that of a lower concentration (2 ng/mL) [Figure 1(b)]. However, the difference in release percentages began to decrease over the experimental period, and showed no statistically significant difference at three weeks of incubation (48% for 20 ng/mL and 41% for 2 ng/mL). This result demonstrated that the initial loading difference played an important role in the release profile of BMP-2 from the chitosan gel, resulting in the concentration gradient driven-diffusion. The result also exhibited a sustained release of BMP-2 over a three-week period.

Cytotoxicity of chitosan gels

The cytotoxicity of the chitosan gels on W-20-17 and HEPM was examined by a MTS assay and a live/dead viability assay. Figure 2 shows photomicrographs of viable W-20-17 and HEPM stained with calcein AM for cell morphologies. The results indicated that the cells encapsulated inside chitosan gels were viable but kept a round shape [Figure 2(a,c)] after three days incubation while cells that settled on the bottom of the well plate proliferated well and displayed in spindle-like shapes [Figure 2(b,d)]. In addition, the effect of the chitosan gels on cell viability via an MTS assay was shown in Figure 3. There were significant increases in activity in W-20-17 and HEPM cells after three days of culture ($p < 0.05$). Both cells showed significantly higher metabolic activity at day 3 than at days 1 and 2 ($p < 0.05$), indicating that the cells were able to grow and proliferate in the presence of the chitosan gels.

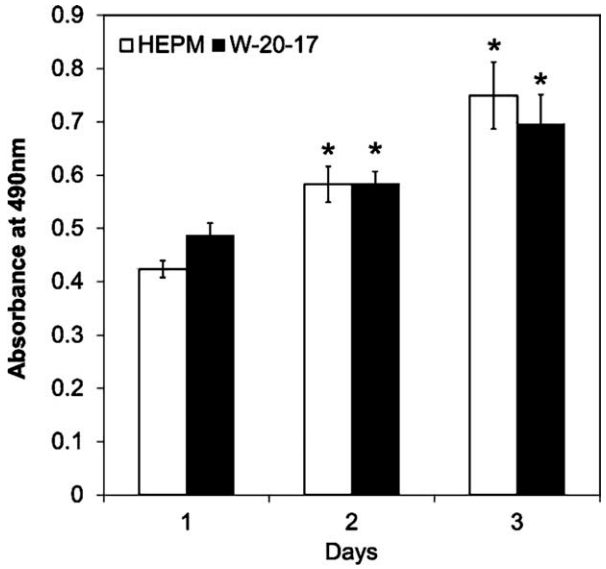

FIGURE 3. Viability of W-20-17 and HEPM cells via indirect culture measured by the MTS assay. The cells cultured on the permeable PET membrane were affected by the chemicals released into the culture media from the chitosan gels. The initial cell density was 35,000 cells per well for 24, 48, and 72 h. The absorbance is expressed as a measure of the cell viability via cell culture media for three days of incubation. Each value represents the mean ± SD ($n = 3$ per each group). *denotes significant difference compared with one day of culture of each cell line ($p < 0.05$).

ALP specific activity

ALP specific activity was assessed as an early indicator of the osteoblastic lineage to study the effect of BMP-2 on osteoblast differentiation. The ALP specific activity was determined at days 7, 14, and 21 of incubation by normalizing the ALP amount to the dsDNA content per sample. Figure 4 shows ALP specific activity of W-20-17 and HEPM at different conditions. ALP specific activity of W-20-17 in all groups peaked at 14 days [Figure 4(a)]. Significant differences in the ALP specific activity were found between the two control groups and the chitosan gel group at 14 and 21 days of incubation ($p < 0.05$). The ALP specific activity of W-20-17 cells in chitosan gel group peaked at day 14 and decreased at day 21. The positive control group showed significantly greater ALP specific activity compared with the negative control group at seven days of culture ($p < 0.05$), indicating the effect of BMP-2 on cell differentiation. However, there were no significant differences between control groups at day 14 and 21. This result indicates that a sustained delivery of BMP-2 using an injectable chitosan gel significantly stimulated ALP activity in W-20-17 for a three-week experimental period.

As shown in Figure 4(b), HEPM exhibited significant increases in ALP activity in all groups at days 14 and 21 of incubation compared to those at day 7 ($p < 0.05$). The positive control group showed significantly higher ALP expression at 14 days incubation compared with the other groups ($p < 0.05$), indicating the induction of osteoblast differentiation by an initial exposure of BMP-2. However, no significant difference was observed between the chitosan gel group and the negative control group at three weeks of incubation. This result suggests that the sustained release of BMP-2 from the chitosan gels did not benefit the ALP specific activity of HEPM at this experimental period.

Alizarin Red S staining for mineralization

To evaluate the effect of BMP-2 treatment on calcium mineral deposition, which is an index of differentiated osteoblast function, cell layers were stained using a 1% Alizarin

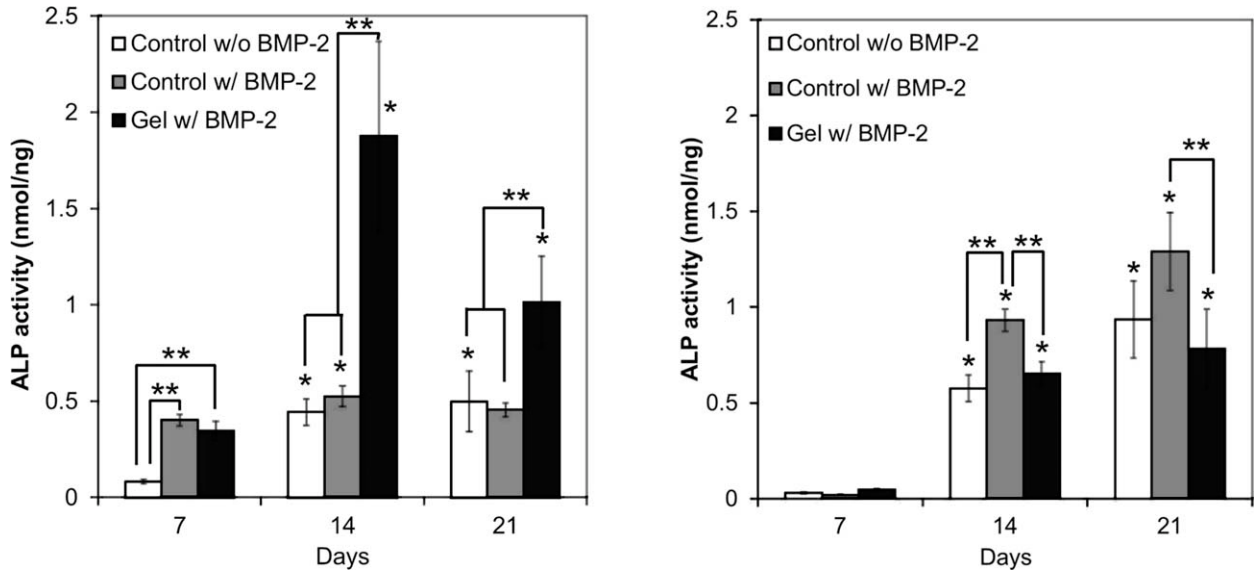

FIGURE 4. The effect of BMP-2 treatment on induction of alkaline phosphatase (ALP) activity in (a) W-20-17 and (b) HEPM cells. Cells were incubated in culture medium without incorporation of BMP-2 (a negative control), with 50 ng/mL of BMP-2 (a positive control), and with the chitosan gel loaded with 50 ng/mL of BMP-2. The ALP activity was determined at 7, 14, and 21 days of cultures and normalized for the dsDNA content. ALP activity is expressed as nmol/ng. Each value represents the mean ± SD ($n = 3$ per each group). *denotes significant difference compared with seven days of culture of each group ($p < 0.05$). **denotes significant difference between groups at each time point ($p < 0.05$).

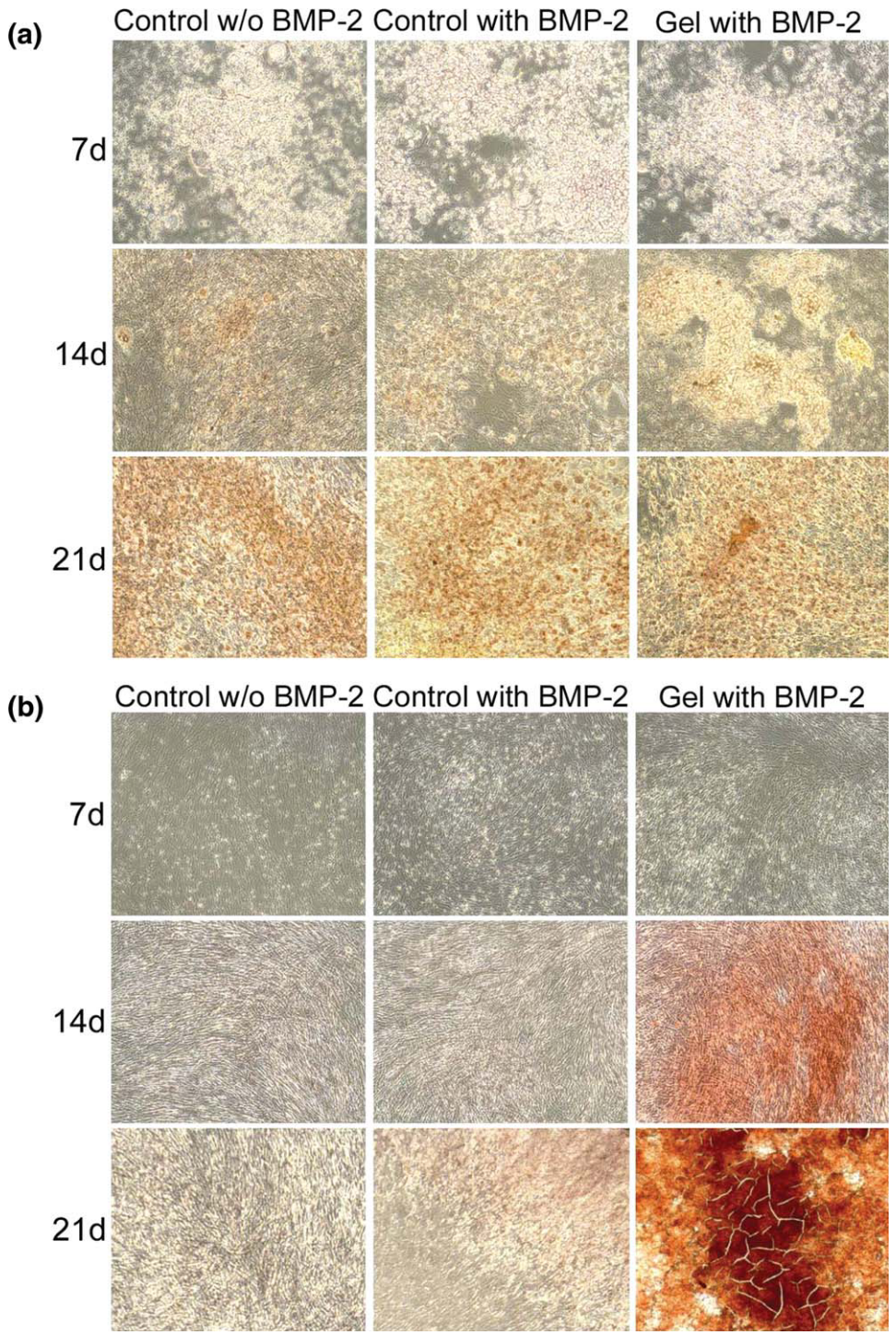

FIGURE 5. The effect of BMP-2 treatment on calcium mineral deposition in (a) W-20-17 and (b) HEPM cells. The presence of mineral within the cell layers was stained with Alizarin Red S (AR-S) staining solution. Cells were incubated in culture medium without the incorporation of BMP-2 (a negative control), with 50 ng/mL of BMP-2 (a positive control), and with the chitosan gel loaded with 50 ng/mL of BMP-2. The AR-S staining was performed at 7, 14, and 21 days of cultures. The pinkish red color demonstrates AR-S positive staining for calcium mineral in the cell layers (MAG = ×100).

Red S (10 mg/mL) staining method. Figure 5 shows the calcium mineral deposition of W-20-17 and HEPM for three weeks of incubation. In the cultures of W-20-17 [Figure 5(a)], cell layers in all groups showed pale pinkish colors at seven days and exhibited more densely stained areas at 14 and 21 days. However, no difference in cell layers stained with Alizarin Red S was observed between groups within the experimental time periods, indicating no enhanced effect of BMP-2 on differentiation of W-20-17 into a mineralized phenotype.

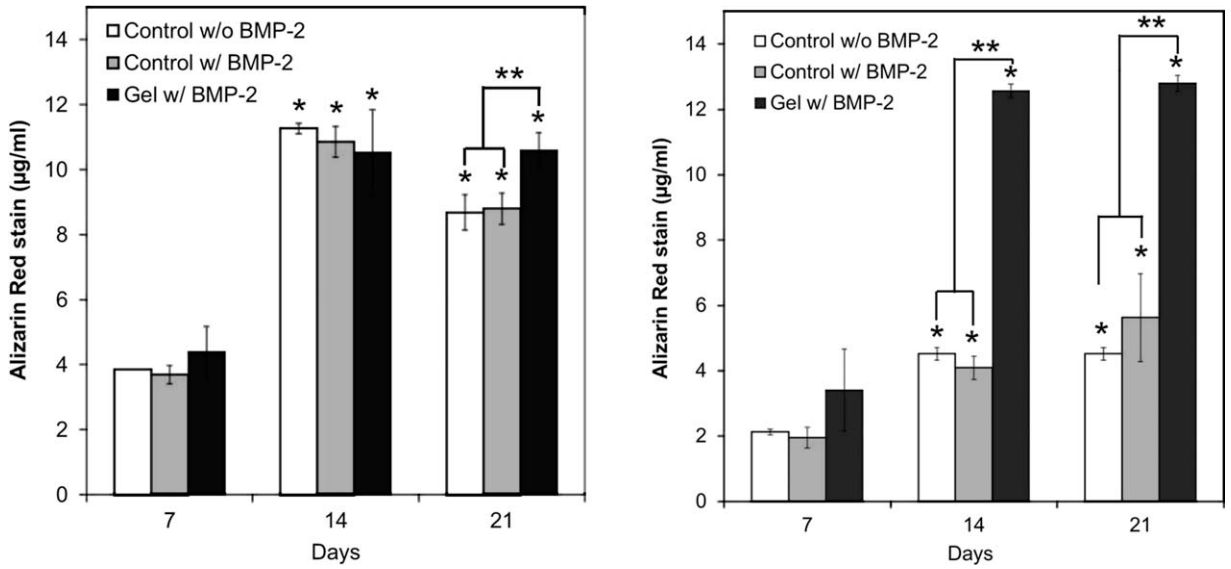

FIGURE 6. The calcium mineral contents quantitatively determined from Alizarin Red S staining extracts from the cell layers of (a) W-20-17 and (b) HEPM. Destained Alizarin Red S concentrations were determined at the absorbance of 450 nm and expressed as μg/mL. Each value represents the mean ± SD ($n = 3$ per each group). *denotes significant difference compared with seven days of culture of each group ($p < 0.05$). **denotes significant difference between groups at each time point ($p < 0.05$).

As shown in Figure 5(b), no calcium mineral deposition of HEPM cells was observed at seven days, suggesting that HEPM cells did not differentiate into a mineralized phenotype in all groups at one week. However, at 14 days, a large cell layer was stained with Alizarin Red S in the chitosan gel group. The orange–red color indicates the slight calcium deposition on the layer of HEPM when cells were treated with a continuous release of BMP-2 from the chitosan gel. Significant calcium deposition of HEPM was observed at 21 days in the chitosan group compared to the other groups. The large nodules stained heavily with Alizarin Red S indicate a more intense pattern of mineral, suggesting that HEPM had the ability to differentiate into osteoblasts due to a sustained BMP-2 treatment. However, cells in the control groups did not exhibit significant calcium deposition due to the lack of a continuous BMP-2 supplementation through the entire culture period.

Figure 6 shows quantitative levels of calcium mineral content measured by a destaining procedure. In W-20-17 cultures [Figure 6(a)], calcium mineralization in the cell layers significantly increased at 14 days compared with at seven days in all groups ($p < 0.05$) but decreased at 21 days in control groups. The result showed that mineral formation of W-20-17 did not seem to depend on the presence of BMP-2 into culture medium, suggesting a BMP-2 independent mineralization characteristic of this cell phenotype. In HEPM cultures [Figure 6(b)], calcium mineral formation in all groups significantly increased at 21 days compared with seven days ($p < 0.05$). In the control groups, calcium deposition increased in a time dependent manner but there was no significant difference between groups. A significant increase in calcium mineral content was observed in the chitosan gel group compared to the control groups at 14 and 21 days ($p < 0.05$). These results suggest that the initial rate of calcium mineral deposition in W-20-17 cells was faster than in HEPM cells in the absence of BMP treatment. However, continuous supplemental BMP-2 to HEPM cells via chitosan gel significantly affected the level of mineral formation.

Osteocalcin production

To test the effect of BMP-2 treatment on osteocalcin expression in the two bone cell lines, an osteocalcin assay was performed with the media sampled at 0-7, 7–14, and 14–21 days. As shown in Figure 7(a), the chitosan gel group exhibited significantly higher osteocalcin expression in W-20-17 compared with the control groups at seven days of incubation ($p < 0.05$). However, W-20-17 in all groups showed low osteocalcin expression, indicating that W-20-17 failed to synthesize osteocalcin throughout the whole culture period.

However, osteocalcin expression of HEPM in all groups [Fig. 7(b)] significantly increased at 14 and 21 days of incubation compared with at seven days ($p < 0.05$). HEPM cells in the chitosan gel group exhibited significantly higher osteocalcin synthesis by approximately 2.3-folds at 14 days compared with control groups ($p < 0.05$), indicating a beneficial effect of sustained release of BMP-2 from the chitosan gel on osteocalcin expression. Osteocalcin expression of HEPM in the chitosan group peaked at 14 days of incubation. An increase in osteocalcin expression is associated with the mineralization period, which is a phase of osteogenesis. These results are consistent with the observations in the Alizarin Red S staining.

DISCUSSION

In bone tissue engineering applications, chitosan is an attractive biopolymer due to its biodegradability and biocompatibility. In particular, chitosan based delivery systems have gained

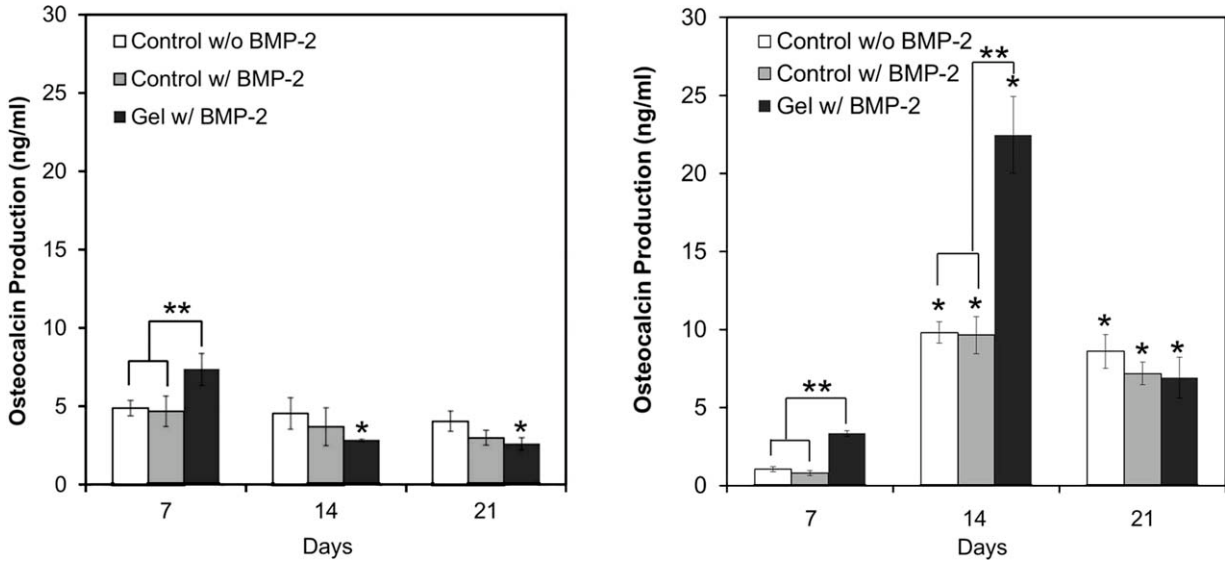

FIGURE 7. The osteocalcin contents secreted from (a) W-20-17 and (b) HEPM cells. Cells were incubated in culture medium without the incorporation of BMP-2 (a negative control), with 50 ng/mL of BMP-2 (a positive control), and with the chitosan gel loaded with 50 ng/mL of BMP-2. Secreted osteocalcin content was measured with the media sampled at 0–7, 7–14, and 14–21 days. The osteocalcin production in cells was expressed as ng/mL. Each value represents the mean ± SD ($n = 3$ per each group). *denotes significant difference compared with seven days of culture of each group ($p < 0.05$). **denotes significant difference between groups at each time point ($p < 0.05$).

popularity due to their ability to sustain a controlled release of drugs at a specific local targeted area.[18,23,25,30,31] Generally, drug release from biodegradable polymeric devices is governed by diffusion, initial drug loading, drug/polymer interactions, drug solubility, and polymer degradation.[41] In this study, the incorporation of BMP-2 into chitosan/β-GP gels showed an initial burst release followed by a sustained release of BMP-2 over a three-week period. This result was consistent with the findings of other studies.[18,21,29] For example, Wu et al. investigated the in vitro release profile of doxorubicin hydrochloride (DX) from an injectable chitosan/β-GP gel.[29] Their release results showed a similar trend that the drug had a high initial burst release followed by a prolonged, slow release This trend was dependent on the pH-sensitivity of the chitosan/β-GP gels. In another study, Ruel-Garie'py et al. demonstrated that the pre-encapsulating drugs into a chitosan/β-GP gel formulation allows for a prolonged release of drugs.[18] As a whole, the evidence suggests that the delivery of drugs and growth factors by injectable chitosan gels is not only initially via diffusion, but also later via degradation of the hydrogel.[18,21,29] In addition, the initial release rate can be controlled by molecular weight, degree of chitosan deacetylation, and concentration of β-GP.[14,18,21–23]

We also investigated the effect of BMP-2 delivered by the chitosan gels or direct addition into medium on cell behaviors of W-20-17 and HEPM. The cell behaviors were characterized using the most well established bone markers, such as the level of ALP activity, calcium mineralization, and osteocalcin production.

First, we evaluated viability of W-20-17 and HEPM cells via direct and indirect cultures with the chitosan gels. Both cells were viable and proliferated over a three-day culture period. In particular, no red stained cells, or dead cells, were observed inside the gels during the three-day incubation. As a whole, our data suggested that the chitosan gels were non-toxic. In order to reduce the cytotoxicity of injectable gel delivery systems, some studies have used low concentrations of inorganic phosphate salts[21,26] or less toxic cross-linkers such as glyoxal and hydroxyethyl cellulose, which are responsible for the solidification of chitosan based hydrogels with low concentrations of β-GP.[18,25] In this study, the acidic chitosan solution was dialyzed prior to adding β-GP, thereby reducing the amount of β-GP needed and improving cell viability.[30]

Osteogenesis consists of three major phases: proliferation, maturation, and mineralization.[35–43] During these periods, specific expressions of bone markers are temporally and sequentially organized. ALP is an early marker of osteoblast differentiation and expressed at high levels at the end of proliferation and during the period of maturation.[1–3,35,37] W-20-17 was derived from mouse bone marrow stroma and has been used as an ASTM standard test for in vitro biological activity of BMP-2. Some studies demonstrated that BMP-2 significantly stimulated ALP activity in W-20-17 in a dose-dependent manner.[44,45] BMP-2 treatment on W-20-17 was shown to reduce the time course of protein synthesis, thereby accelerating ALP production.[44] Kempen et al. demonstrated that the bioactivity of the BMP-2 delivered by composite materials was higher than similar concentrations of BMP-2 added directly to the W-20-17 cultures, suggesting that BMP-2 is susceptible to degradation in the culture medium over a seven-day culture period.[45] Similarly, we also found that the W-20-17 significantly increased ALP activity in response to a sustained BMP-2 treatment via the chitosan gel delivery system. The W-20-17 in the presence of the chitosan gel group expressed higher ALP activity by ~3.6- and 2.1-folds compared with control groups at 14 and 21 days, respectively [Fig. 4(a)]. The positive control group exhibited

significantly higher ALP activity at seven days compared to the negative control group ($p < 0.05$). However, there was no significant difference between control groups at 14 and 21 days. This result was consistent with previous studies indicating that constant supplementation of BMP-2 significantly affects bone cell differentiation.[39,42,45] Therefore, the higher ALP activity in the chitosan gel group is probably due to the sustained release of BMP-2 from the chitosan gel, which maintains BMP-2 concentrations at sufficient levels over the culture period.

HEPM is a human embryonic palatal mesenchymal cell line. ALP activity of HEPM did not significantly increase in response to a sustained treatment of BMP-2. The findings from this study are consistent with the fact that cell behavior and efficacy of BMP-2 are in a species-dependent manner.[46,47] Some studies demonstrated that ALP activity of human mesenchymal cells remained lower sensitivity to BMP-2 than that of bone marrow stromal cells (MSC) from rats and mice.[46,47] But the expression of other genes such as noggin, BMP-2, osteopontin, and bone sialoprotein (BSP) were significantly activated when human mesenchymal cells were treated with BMP-2, BMP-4, or BMP-7.[46–49] It is not clear why the alkaline phosphatase gene is resistant to BMP regulation in human mesenchymal cells.[47,48] However, it was reported that human mesenchymal cells pre-treated with dexamethasone showed elevated ALP activity in response to BMP-2.[46–48]

Osteocalcin, a bone specific protein, is produced in clusters of mineralized cells and plays a role in bone resorption [35–37,44]. Previously, Thies et al. investigated the effect of BMP-2 on mineralization and osteocalcin synthesis of W-20-17 cells. Their results showed that W-20-17 cells failed to mineralize both *in vitro* and *in vivo* even though the cells were supplemented with 100 ng/mL BMP-2, 50 μ/mL ascorbic acid, and 7 m*M* β-glycerophosphate for up to 10 days.[44] However, W-20-17 cells expressed osteocalcin positively after four days treatment in a dose-dependent manner. Similarly, in our study, W-20-17 cells showed significantly higher osteocalcin expression at seven days in the chitosan group, but maintained a low level of osteocalcin expression over a three-week period. In addition, our Alizarin Red S stain test showed that mineral formation was not dependent on the presence of BMP-2 in W-20-17 cultures, but the initial rate of calcium mineral deposition was faster than that of HEPM cultures (Figure 6).

On the other hand, a continuous supplementation of BMP-2 from the chitosan gels could increase calcium mineral deposition on the layer of HEPM. HEPM cells expressed significantly higher osteocalcin synthesis in the chitosan group at 7 and 14 days compared with control groups ($p < 0.05$). This study indicates the difference in the ability of the two different cell lines to undergo osteogenesis in response to BMP-2.

During bone formation, different stages of mineralization and subsequent development of mineral phases are strongly associated with interactions between organic and inorganic molecules.[37] If osteogenic sources are present in the cell culture, for example, mineralization is rapidly initiated and enhanced to form apatite-like calcium mineral depositions. It has been suggested that ascorbic acid, β-glycerophosphate (β-GP), and vitamin D_3 are essential to support bone matrix deposition and mineralization.[35–43] In this study, β-GP was added into the chitosan solution to induce a thermo-sensitive chitosan gel at body temperature. It is likely that the release of organophosphates from the chitosan gel might take part in significantly higher calcium deposition levels by HEPM in the chitosan gel group. Several studies emphasized that various phosphate agents could stimulate a rapid and highly reproducible formation of mineral phages in a dose and time dependent manner.[37–43] Therefore, a sustained local delivery of phosphate ions can increase mineralization in a controlled manner *in vivo*.[38] However, in this study, the potential mineral stimulating effect by organophosphates was not observed on the W-20-17 cells, suggesting species dependent behaviors.

Our findings from this study showed the potential advantages of the injectable chitosan gel for a local delivery of BMP-2 to induce osteoblast differentiation. These results suggest to some extent the beneficial effect of BMP-2 released from chitosan gels on osteoblastic differentiation of W-20-17 and mineralization of HEPM. It might be desirable to investigate the thermal gelation, release profile, and biological efficacy of the gels with different concentrations of β-GP. It is worth noting that a chitosan/β-GP gel delivery system potentially represents a new strategy for facilitating mineral formation by the combination of a sustained local delivery of BMP-2 with a sustained release of phosphate groups.

CONCLUSIONS

This study investigated the effect of BMP-2 supplementation on osteoblast differentiation using a non-surgical local delivery system. The *in vitro* release profile of BMP-2 showed a sustained release from the chitosan gel over a three-week experimental period. Two cell lines (W-20-17 and HEPM) cultured in the chitosan gels were viable and were able to proliferate. The enhancement effects of BMP-2 released from the chitosan gels on osteogenic differentiation and mineralization are species and cell type dependent. It has been suggested that W-20-17 is suitable for initial ALP response, and HEPM is suitable for mineralization study.

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