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Effects of simvastatin-loaded polymeric micelles on human osteoblast-like MG-63 cells

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ABSTRACT

To develop an optimized simvastatin (SV) delivery device for bone regeneration, SV-loaded poly(ethylene glycol)-poly(ε-caprolactone) (PECL) micelles were constructed. The micelles had an average size of 80 nm. The *in vitro* release behavior of SV from the micelles showed prolonged release compared to the free SV. The following four groups were tested in a cytologic experiment: a free SV group, a SV-loaded micelle group with SV concentrations ranging from 2.5×10^{-6} to 2.5×10^{-10} M, a drug-free micelle group and a blank control group. The effect of SV-loaded micelles on osteoblast-like MG-63 cells was determined *via* analysis of cell proliferation, alkaline phosphatase activity, and cell calcification. In addition, the mRNA and protein expression of the BMP-2 gene were determined with real-time fluorescence quantitative polymerase chain reaction and western blot techniques, respectively. The results show that SV-loaded PECL micelles cause effective suppression of the osteoblast early proliferation inhibition, stimulation of osteoblast differentiation and mineralization, and stimulation of the BMP-2 expression. Therefore, SV-loaded PECL micelles are predicted to have great potential in bone regeneration applications.

1. Introduction

Bone loss diseases, including osteoporosis, remain a major public health problem [1]. Since Mundy et al. [2] found that statins, the widely used, lipid-lowering hydroxymethylglutaryl-coenzyme A reductase inhibitors, had an anabolic effect on bone formation both *in vitro* and *in vivo* through activation of the BMP-2 gene promoter, increasing numbers of studies have verified the beneficial effect of statins on bone formation [3,4], reduction of fracture risk [5,6] and enhancement of bone mineral density [7–9]. Because of their dual anabolic and antiresorptive actions, statins represent one of the most promising families of anti-osteoporotic drugs [10]. Simvastatin (SV), a well-known member of the statin family, has been safely administered orally for over 20 years. It acts to reduce serum cholesterol levels and the subsequent risk of heart attack, and it also has very few side effects. For bone loss diseases, efficacy is not expected with routine oral administration of SV doses because the ultimate systemic availability is only 2.4% [11] and is most likely far less in the *in vivo* bone microenvironment. However, it has been observed that with the local application of SV at healing sites, there is a rapid dispersion, a short half-life and few some side effects associated with SV, such as cytotoxic [12]. Therefore, delivery systems have gained considerable attention in attempts to bridge the gap between research and clinical applications.

In previous studies, Shah and Pathak [13] described the controlled release kinetics of SV from solid lipid nanoparticles (mean particle size ~200 or ~300 nm) *in vitro*. Tanigo et al. [14] developed a gelatin hydrogel that incorporated water-insoluble SV and obtained sustained release of the drug from this hydrogel. Yoshii et al. [15] constructed polyurethane scaffolds containing lovastatin (LV) and demonstrated that locally delivered LV enhanced *in vivo* new bone formation with the scaffolds after 4 weeks. Despite these promising results, research into delivery systems for SV is still at an early stage and powerful delivery formulations are still required clinically for oral administration, intravenous injection and local implantation.

The polymeric micellar system is considered to be a promising method for enhancing the bioavailability and reducing the toxic side effects of hydrophobic drugs because it has significant benefits in terms of drug loading, release, delivery, and stability [16]. The

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polymeric micelle system has a unique core–shell architecture in which the hydrophobic core serves as a reservoir for hydrophobic drugs. This hydrophobic core can protect drugs from decomposition and control their release, while the hydrophilic shell imparts steric stabilization in physiological fluids and enables active targeting. Because of the unique core–shell architecture and small size (diameter usually less than 100 nm), such micelles are not susceptible to recognition and uptake by the reticuloendothelial system (RES), hepatic excretion, or renal excretion [17,18]. Therefore, they are normally used as sustained-release drug carriers to prolong the circulation time of the drug in the blood and improve bioavailability. Moreover, the potential use of these micelles for the production of drug formulations with improved oral bioavailability has been recently reported [19]. In addition to their use alone, polymeric micelles can be incorporated into scaffolds for broader applications.

In the present work, the major objective was to construct SV-loaded poly (ethylene glycol)-poly (ε-caprolactone) (PECL) micelles and evaluate the effect of the SV delivery formulation on osteoblast proliferation, differentiation, mineralization and BMP-2 gene expression. The delivery system presented here is composed of PECL micelles, in which the hydrophobic block poly (ε-caprolactone) (PCL) is the core SV reservoir, and the hydrophilic block poly(ethylene glycol) (PEG) is the hydrophilic shell that avoids non-specific absorption by the RES [17]. To determine the effect of these micelles on osteoblast-like MG-63 cells, the cell proliferation, ALP activity, cell calcification, and mRNA and protein expression of BMP-2 in these cells were investigated. These studies help to explore the potential use of these micelles in bone regeneration applications. We chose the PECL copolymer as the delivery system because of its biocompatibility, slow degradation rate, ease of processing, and demonstrated use in bone tissue engineering [20,21]. The PECL copolymer was used to prepare the drug-loaded micelles by the direct entrapment of a drug into the micelle core without the covalent attachment of the drug molecules to the core-forming blocks. It has been reported that such PECL co-polymer micelles were successfully used as delivery vehicles for dihydrotestosterone [22], with good compatibility between the core-forming block, PCL, and the incorporated drug, dihydrotestosterone. However, the compatibility between the PCL block and SV has yet to be examined, which is the reason for the present study. It was expected that an enhanced bioavailability of SV for bone formation could be obtained because the micellar delivery system can prolong the blood circulation time of SV, which would ensure the transport of SV to the bone microenvironment by oral administration, intravenous injection or local implantation.

2. Materials and methods

2.1. Preparation of PECL micelles

SV-loaded micelles were prepared by a membrane dialysis method. Briefly, 100 mg of PECL (PEG: Mn = 2000 g mol^{-1}, PCL: Mn = 2000 g mol^{-1}) (Jinan Daigang Biomaterial Co., Ltd., Jinan, China) and a fixed amount of SV were dissolved in 16 mL of acetone. De-ionized (DI) water (8 mL) was added dropwise to the polymer solution while stirring, followed by ultrasonication in an ice bath. This mixture was then dialyzed against deionized water at room temperature for 24 h using a dialysis bag with a molecular weight cut-off of 3500 g mol^{-1}. The dialysis process enabled the solvent exchange between the acetone and deionized water, during which, self-assembly of the PECL occurred. After dialysis, the solution in the dialysis bag was collected and filtered through a 0.45-μm syringe filter. As a control, PECL micelles without SV were also prepared.

2.2. Characterization

The particle sizes of the PECL micelles and SV-loaded PECL micelles were evaluated by dynamic light scattering (Nano ZS, ZEN3600, Malvern Instruments Ltd., Malvern, UK) after appropriate dilution with DI water. Light scattering data were obtained at a detection angle of 173° at 25 °C and were subsequently analyzed by the cumulant method to obtain the hydrodynamic diameter and polydispersity index (PDI) of the micelles. The morphology of the micelles was investigated using atomic force microscopy (AFM; Agilent 5500 series, Agilent Technologies Inc., Santa Clara, USA) by applying the tapping mode. Before observation, a drop of the micelle solution was deposited onto a silicon chip and then freeze-dried.

2.3. Encapsulation ratio and drug-loading efficiency

To determine the SV encapsulation ratio and drug-loading efficiency, a known amount of SV-loaded micelles was dissolved in 1 mL of tetrahydrofuran (THF). This solution was then analyzed by an ultraviolet (UV) spectrophotometer at 238 nm with PECL solution as the control. The drug concentration in the solution was calculated based on the standard curve. The drug encapsulation efficiency (EE) and drug-loading ratio (DL) were obtained in accordance with the following formulae:

$$\mathrm{EE} = \left(\frac{\text{amount of drug loaded in the micelles}}{\text{amount of drug added during fabrication}}\right) \times 100\%$$

$$\mathrm{DL} = \left(\frac{\text{amount of drug loaded in the micelles}}{\text{the total amount of drug in the micelles and PECL conjugate used in the process}}\right) \times 100\%$$

2.4. In vitro SV release

Three groups of micelles were loaded with 200 μg, 400 μg or 600 μg of SV. The same amounts of free SV were prepared as controls. Both the SV-loaded micelles and the free SV were placed into the dialysis bag suspended in a 0.2% SDS solution. The total volume of each sample solution was 20 mL. The sealed vials were placed in a gas bath thermostatic oscillator (SHZ-82, Jintan Honghua Instruments Co. Ltd., Jintan, China) at 75 rpm and 37 °C. At predetermined time intervals, 3 mL of the solution was withdrawn from the release medium and replaced with fresh medium. The SV concentration was analyzed by measuring the UV absorbance at 238 nm.

2.5. Cell culture

The cytologic features of the SV-loaded PECL micelles were evaluated *in vitro* using human osteoblast-like MG-63 cells. MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Life Technologies Corp., NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco Laboratories, Life Technologies Corp., NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma–Aldrich Corp., St. Louis, USA), under standard culture conditions (37 °C and 5% CO_2). The medium was refreshed twice a week.

The following four groups were tested: SV-loaded PECL micelles (SVPM), free SV (FSV) as the positive control, PECL micelles (PM) and a blank (BK), as the negative control. MG-63 cells in log growth phase were plated at a density of 2.0×10^4/well in 200 μL of medium in 96-well plates. After 24 h of culture, during which time the cells attached to the plate, the PECL micelles, SV-loaded PECL

micelles and free SV at concentrations ranging from 2.5×10^{-6} to 2.5×10^{-10} M were added to the medium for the PM, SVPM and FSV groups, respectively.

2.5.1. Cell proliferation

Cell proliferation was measured with the (3-(4, 5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium (WST) assay using a Cell Counting Kit-8 (Dojindo, Japan). In this assay, metabolically active cells react with the tetrazolium salt in the WST reagent to produce a soluble, formozan dye. The cells were cultured with the different drug formulations for 24, 48 and 72 h, followed by the addition of 10% cck-8 reagent. After an additional 4 h incubation at 37 °C, the absorbance at 450 nm in each well was measured with a Microplate Reader (Multiscan MK3, Thermo Fisher Scientific Inc., Hudson, USA).

2.5.2. Alkaline phosphatase activity

Alkaline phosphatase (ALP) produced by the cells was measured to determine the osteoblastic functional activity. After 48 h in culture, the cells were harvested and subjected to a cyclic freezing/thawing process. The cell lysates were quantified with a colorimetric quantitative assay kit (GenMed, Shanghai, China) using p-nitrophenylphosphate (pNPP) as the substrate. In the presence of magnesium ions, pNPP was hydrolyzed by the phosphatases to phosphate and p-nitrophenol. The rate of p-nitrophenol liberation is proportional to the ALP activity and can be measured photometrically. Therefore, the absorbance of the solution at 405 nm was measured, and the amount of ALP was then calculated. The protein concentrations were evaluated by the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific Inc., Hudson, USA). The absorbance of the solution was measured at 562 nm, and the amount of total protein was calculated using a standard curve. To determine the ALP activity, the amount of ALP was normalized to the amount of total proteins synthesized.

2.5.3. Mineralization assay

Alizarin Red S staining was performed to detect mineralization. Alizarin Red S dye selectively binds to calcium salt and has been widely used for calcium mineral histochemistry. After 7d in culture, the cells were washed with PBS and fixed in ice-cold 70% ethanol for 1 h, followed by staining with Alizarin Red S (40 mM, pH 4.2) for 1 h. After extensive washes with water, these specimens were observed under an optical microscope. The optical density of staining was analyzed by computerized image pattern analysis.

2.5.4. Measurement of BMP-2 gene expression by FQ-PCR analysis

FQ-PCR, which can accurately measure the mRNA level of a gene, is highly reliable for *in vitro* studies. Although posttranslational modifications and regulation may occur, the amount of mRNA may be considered a good predictor of the protein level. After the MG-63 cells of each group were cultured for 48 h, the BMP-2 RNA was extracted with the TRizol Reagent (Invitrogen Laboratories, Life Technologies Corp., NY, USA), followed by chloroform purification and isopropanol precipitation. The purity of the RNA was verified by measuring the OD 260/OD 280 absorbance ratio using a Biophotometer Plus (Eppendorf, Hamburg, Germany), and ensuring that this value was 1.8. The integrity of the RNA was verified by sepharose electrophoresis. The reaction reagents for RNA reverse transcription were added to a PCR tube. The final reaction volume for RNA reverse transcription was 20 μL, consisting of 1 μg total RNA, 0.5 μL oligo(dT), 0.5 μL random primer, 0.5 μL RNase inhibitor, 0.5 μL MMLV, 2 μL dNTPs, 4 μL 5× buffer, and DEPC-treated water. Reverse transcription was performed at 30 °C for 10 min, followed by 42 °C for 60 min. The reaction was then denaturated at 72 °C for 10 min.

The quantitative PCR resulted in a 280 bp amplicon of BMP-2 produced by a BMP-2 primer pair (forward, 5′-ACGTCAAGCCAAACACAAAC-3′; reverse, 5′-AAGGTACAGCATCGAGATAGC-3′). A second amplicon of 112 bp was produced with an 18S rRNA primer pair (forward, 5′-CCTGGATACCGCAGCTAGGA-3′; reverse, 5′-GCGGCGCAATACGAATGCCCC-3′) located within the BMP-2 amplicon as the reference. The PCR reaction volume was 20 μL and consisted of the following: 5 μL cDNA (1/20 dilution), 0.5 μL BMP-2 primer, 0.5 μL 18S rRNA primer, 10 μL 2× SYBR Green PCR Master Mix, and 4 μL DEPC-treated water. The reactions were performed in a PCR amplification machine (ABI7500, Applied Biosystems, Foster City, USA). The amplifying conditions were as follows: pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 32 s. A single fluorescent reading was taken at the end of each cycle. The specificity of the primers was confirmed for every PCR run by melting curve analysis.

2.5.5. Western blot analysis

Western blotting was used to measure the protein expression of BMP-2 in MG-63 cells cultured for 48 h with the different drug formulations. Cells were washed with ice-cold PBS 3 times and lysed with a lysis buffer containing protease inhibitors. The protein content was quantified using the BCA protein assay. Equal amounts of protein (30 μg per lane) were loaded onto a 10% SDS-PAGE gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h and then incubated in the primary antibody in TBST (1/300 dilution) at 4 °C overnight. Following incubation with the primary antibody, the membrane was washed and incubated with goat anti-rabbit IgG/Alexa Fluor 350 for 1 h at room temperature. BandScan 5.0 software was used with a gray scale scan to evaluate the relative values for protein expression.

2.6. Statistical analysis

All data were expressed as the mean ± SD (standard deviation). Statistical analysis was performed with factorial-designed ANOVA and one-way ANOVA using the SPSS statistical package 13.0 (© 2004 by SPSS Inc.). The Levene test for homogeneity of variance was used, followed by a pairwise comparison by the Bonferroni test in the case of homogeneity of variance or by the Tamhane's T2 test in the case of heterogeneity of variance. Differences were considered significant when the *P*-value was less than 0.05.

3. Results

3.1. Characterization of copolymer micelles

Table 1 lists the hydrodynamic diameter and PDI of micelles from different ratios of SV/PECL. The mean diameter of the micelles produced from PECL was approximately 61 nm with a narrow distribution in size. The SV-loading caused an increase in size, most likely because of a change in the micellar structure caused by the SV enclosed in the hydrophobic core of the micelles. The

Table 1
Hydrodynamic diameter and PDI of micelles from different ratios of SV/PECL (mean ± SD, $n = 3$).

Ratio (SV:PECL)	Hydrodynamic diameter (mean ± SD (nm))	PDI (mean ± SD)
0:100	61.3 ± 0.7	0.284 ± 0.018
10:100	86.0 ± 3.8	0.370 ± 0.008
20:100	98.4 ± 0.3	0.375 ± 0.012

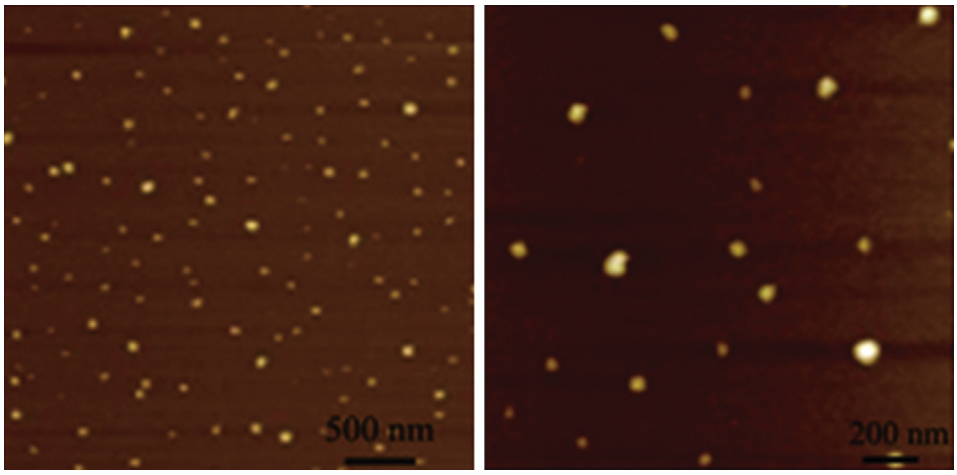

Fig. 1. AFM photographs of SV-loaded PECL micelles.

mean diameter of the micelles produced from 10% of SV/PECL was approximately 86 nm, and those from 20% of SV/PECL were approximately 98 nm. In addition, there was no obvious change in the narrow size distributions of micelles with SV loading. AFM images of SV-loaded micelles are shown in Fig. 1. The micelles were sparsely scattered on the silica surface and were nearly spherical in shape. The micelle sizes in the AFM were uneven, at approximately 80 nm, which is consistent with the DLS determination.

In this study, SV-loaded PECL micelles produced from 10% of SV/PECL with EE and DL of 78.94 ± 11.51% and 7.89 ± 1.15%, respectively, were selected for the *in vitro* release test and cytologic experiments. These EE and DL values accounted for the high loading capacity of PECL micelles.

3.2. In vitro release of SV from drug-loaded micelles

The *in vitro* drug release study was performed in 0.2% SDS at 37 °C. The cumulative release profiles of SV from drug-loaded micelles are shown in Fig. 2. A typical two-phase release profile was observed. This means that there was an initial relatively rapid release of the drug followed by a sustained and slow release of the drug over a prolonged period of time. In the initial 10 h, 73, 36 and 31% of the incorporated drug was released, and 100, 74 and 56% was released within 60 h, 84 h and 84 h for micelles loaded with 200 μg, 400 μg and 600 μg of SV, respectively. The equivalent amount of free SV released was almost 100, 73 and 57% within 12, 36 and 60 h, respectively.

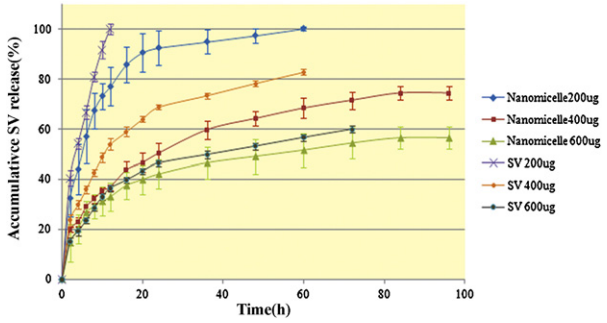

Fig. 2. *In vitro* drug release profiles of SV-loaded PECL micelles with 200, 400 and 600 μg of SV, and free SV at the equivalent amount (means ± SD, $n = 3$).

3.3. Cell proliferation

Fig. 3 shows the proliferation of MG-63 cells incubated with the drug formulations for 24, 48 and 72 h. The WST assay indicated that the number of cells increased significantly from 24 to 72 h, suggesting that the cells proliferated in all groups (SVPM, FSV, PM and BK). However, a statistically significant decrease in cell proliferation was observed at 24 h for all concentrations of SV except 2.5×10^{-8} M in the SVPM group and all concentrations of SV except 2.5×10^{-9} M in the FSV and PM groups, compared to the BK group. In contrast, cell proliferation in the SVPM group was significantly higher compared to the FSV group at the equivalent SV concentration of 2.5×10^{-7} M. This result suggests that a certain amount of cytotoxicity may be caused by high dose SV during the early stages of proliferation and that the cytotoxicity may be reduced when SV is delivered by sustained release from the SV-loaded PECL micelles.

3.4. ALP activity

The osteoblastic differentiation of the MG-63 cells was evaluated by measuring ALP activity after culture with the drug formulations for 48 h. As shown in Fig. 4, the ALP synthesized by the cells at a SV concentration of 2.5×10^{-7} M in the FSV group was significantly higher than that observed in the BK group. ALP was more highly expressed by cells in the SVPM group with SV concentrations ranging from 2.5×10^{-6} to 2.5×10^{-10} M than that observed

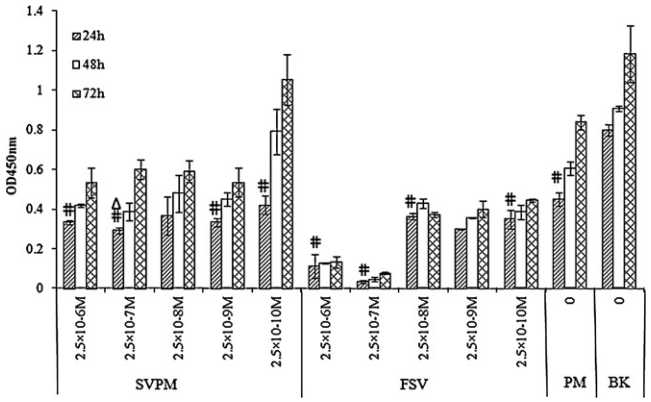

Fig. 3. Proliferation of MG-63 cells cultured with SVPM, FSV, PM or BK for 24, 48 and 72 h. (mean ± SD, $n = 3$; $^{\#}P < 0.05$ *vs.* BK; $^{\Delta}P < 0.05$ *vs.* FSV with the equivalent SV concentration).

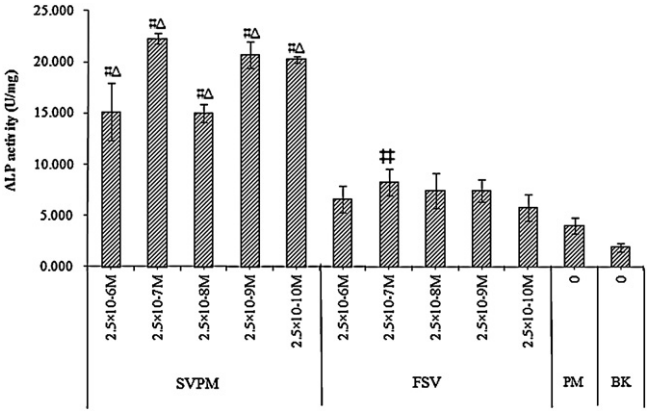

Fig. 4. ALP activity of MG-63 cells after 48 h of culture with SVPM, FSV, PM or BK (mean ± SD, $n = 3$; $^{\#}P < 0.05$ *vs.* BK; $^{\Delta}P < 0.05$ *vs.* FSV with the equivalent SV concentration).

in cells of the FSV group at the equivalent SV concentration, as well as compared to the PM and BK groups.

3.5. Mineral deposition

Bone nodule formation is a specific marker for bone cell differentiation. Alizarin Red S staining was used to characterize the mineral deposition after 7 days of culture with the drug formulations. Fig. 5A shows the photographic images of Alizarin Red S staining of cells treated with SV-loaded micelles with 2.5×10^{-7} M SV. Please note the appearance of reddish-orange mineralized nodules. Quantitative analysis was performed by determining the mineralized area. As indicated in Fig. 5B, the mineralized area of the cells treated with SV-loaded micelles with 2.5×10^{-7} M of SV was significantly larger than that observed in the BK and PM groups.

3.6. The mRNA level of the BMP-2 gene

The mRNA expression level of the BMP-2 gene was evaluated by relative quantification analysis. Two amplification curves and two melting curves were generated from two primer sets (BMP-2 and 18srRNA). Ct values were obtained by amplification curve analysis. The relative expression levels of the BMP-2 mRNA were calculated from samples of MG-63 cells grown in a single culture on tissue culture plastic as a calibrator (Fig. 6). The mean BMP-2 gene expression in the cells of all the groups (SVPM, FSV, PM and BK) were significantly different. Nevertheless, because of the heterogeneity of variance, differences in mRNA expression were not considered significant for pairwise comparison among each concentration of SV in SVPM and FSV, and PM and BK. These values were quite dissimilar to the mean gene expression shown in Fig. 6. The expression of BMP-2 in these cells confirmed the osteoblastic phenotype of the cells.

$$\Delta Ct_{(\text{test})} = Ct_{(\text{target,test})} - Ct_{(\text{ref,test})}$$

$$\Delta Ct_{(\text{calibrator})} = Ct_{(\text{target,calibrator})} - Ct_{(\text{ref,calibrator})}$$

$$\Delta\Delta Ct = Ct_{(\text{test})} - Ct_{(\text{calibrator})}$$

$$\text{Relative expression value} = 2^{(-\Delta\Delta Ct)}$$

3.7. Protein level of BMP-2 gene

The protein expression level of BMP-2 in MG-63 cells cultured with various concentrations of SV was determined by western blot analysis. The photodensity scanning profiles of SDS-PAGE for BMP-2 (Fig. 7A) indicate that the greatest photodensity was shown in SVPM. According to densitometry of the bands, BMP-2 normalized to the GAPDH bands are represented in Fig. 7B. The protein expression of BMP-2 in the SVPM group at each concentration and in the

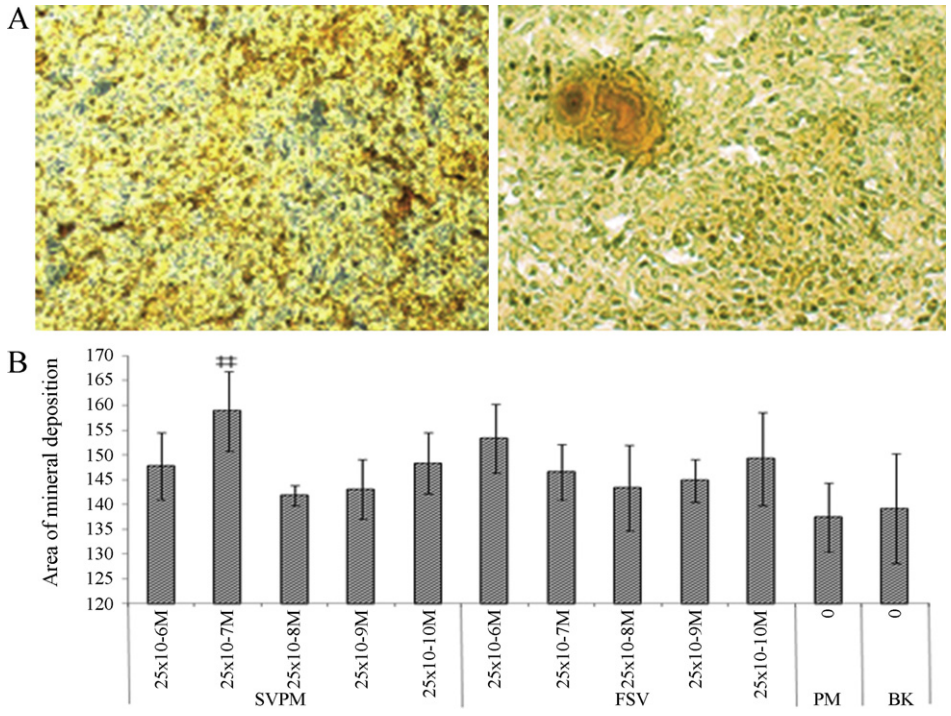

Fig. 5. (A) Photographs of mineral deposition visualized by Alizarin Red S staining. Cells were cultured for 7 days with SVPM with a SV concentration of 2.5×10^{-7} M (100×); (B) The area of mineral deposition determined by analysis of optic density of Alizarin Red S staining after 7 days of culture with SVPM, FSV, PM or BK (mean ± SD, $n = 4$; $^{\#}P < 0.05$ *vs.* BK).

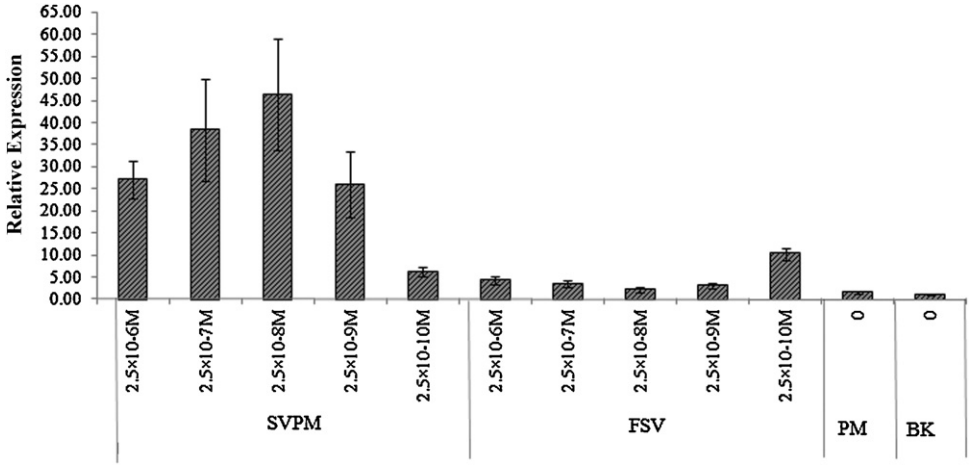

Fig. 6. Relative expression of the BMP-2 gene in MG-63 cells after 48 h of culture with SVPM, FSV, PM or BK (mean ± SD, $n = 3$).

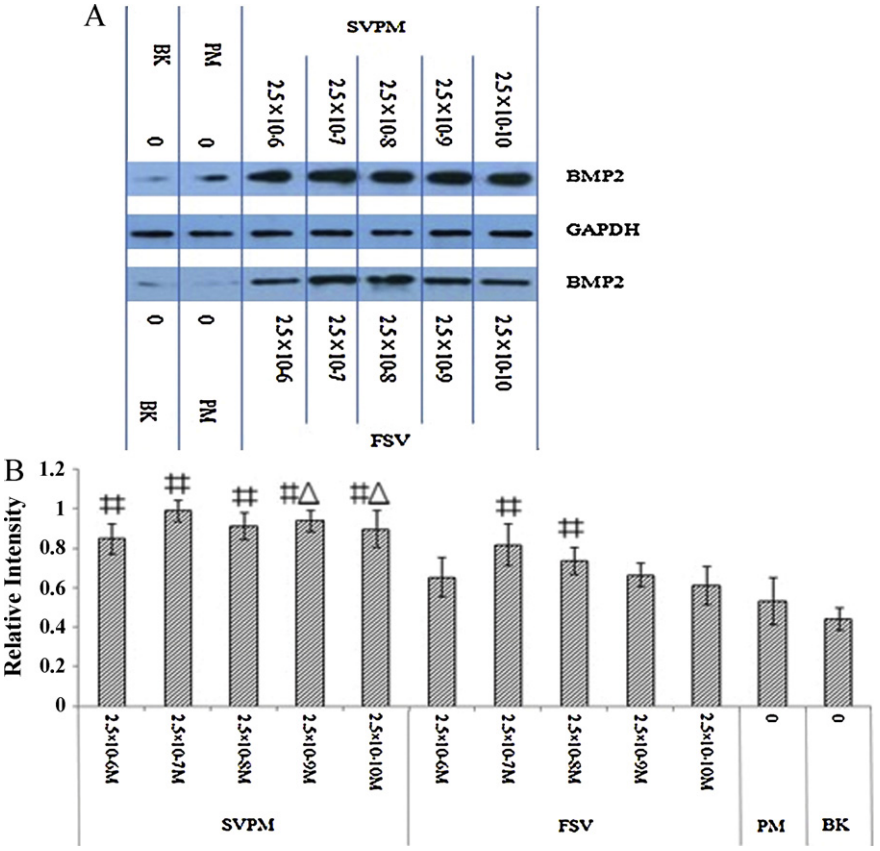

Fig. 7. (A) Western blots of protein expression of BMP-2 in MG-63 cells after 48 h of culture with SVPM, FSV, PM or BK. GAPDH band was the calibration band. (B) The relative intensity of protein expression for BMP-2 after 48 h of culture with SVPM, FSV, PM and BK (mean ± SD, $n = 3$; $^{\#}P < 0.05$ *vs.* BK; $^{\Delta}P < 0.05$ *vs.* FSV with the equivalent SV concentration).

FSV group at 2.5×10^{-7} and 2.5×10^{-8} M of SV were significantly higher than that observed in the BK group. There were significant differences between the SVPM groups and the FSV groups with 2.5×10^{-9} and 2.5×10^{-10} M of SV.

4. Discussion

Statins have been reported to effectively treat bone injuries; however, it has been a great challenge to control the delivery of statins because statins are not very effective in stimulating bone formation when administered orally. In this study, a delivery device for SV was constructed through the incorporation of SV into PECL micelles by the self-assembly of the PECL copolymer. *In vitro* release of SV from PECL micelles showed prolonged release profiles, which accounted for the sustained release effects of the micelles that may improve drug stability with regard to hydrolysis under physiological conditions. Good compatibility between the core-forming block, PCL, and the incorporated drug, SV, was present, based on the high loading capacity of the PECL micelles. During the *in vitro* release of SV, the similar maximal accumulative releases rates of both SV-loaded micelles and free SV at the same concentration, indicated

that the release kinetics of both formulations are controlled by the state of SV dissolution in 0.2% SDS. It appeared that the high filter pressures of SV between the inside and outside of the micelles, which were caused by the state of SV dissolution in 20 mL medium solutions, was beneficial for the release of SV and the degradation of the micelle. SV was completely liberated from micelles loaded with 200 μg SV, and only partly liberated from micelles loaded with 400 μg and 600 μg of SV. This suggested that 400 μg or 600 μg of SV in 20 mL medium solutions may be in an equilibrium of dissolution and precipitation at some point during the test, and 200 μg of SV in the medium solutions may be far from equilibrium.

It has been demonstrated that the SV concentration observed in the serum during clinical treatment commonly ranges from 2.9×10^{-8} to 4.4×10^{-8} M (12.7–18.1 ng/mL) [23]. A 2.7-fold increase in BMP-2 production was reported for MG-63 cells treated with 2.5×10^{-6} M SV [2]. Therefore, in this study, the SV concentration in the cell culture experiments was fixed between 2.5×10^{-10} M and 2.5×10^{-6} M. However, the SV concentrations for the *in vitro* SV release assay from drug-loaded micelles were based on the dosage used for local injection of SV [24].

Cytologic experiments were performed to verify the biological efficacy of SV-loaded PECL micelles. Certainly, the osteoblast is one of the key cells involved in bone metabolism and plays an important role in bone growth, recovery, and maintenance of bone mass. The human osteoblast-like cell line MG-63 is an ideal model for osteoblast cells because it displays many osteoblastic traits common to immature osteoblasts and has a more homogeneous cell population than primary cell cultures of osteoblasts. The cell proliferation test indicated that cytotoxicity was present during the early stages of proliferation with a high dose of SV, and intriguingly, that this cytotoxicity could be reduced by the sustained release of SV from the SV-loaded PECL micelles. ALP activity, an early marker of osteoblast differentiation, indicated that the osteoblast differentiation stimulated by SV-loaded micelles was far superior to that by free SV. Moreover, large mineralized nodules were formed in the MG-63 cells stimulated with SV-loaded micelles with 2.5×10^{-7} M of SV, suggesting that SV-loaded micelles with a suitable concentration of SV may not only promote the initiation of mineral deposition, but may also accelerate the progress of mineralization and cause mature mineralized nodules to appear earlier.

The detailed mechanism of action of SV in inducing bone formation has not been fully understood. The osteoblast-differentiating effect may be explained by a BMP-2-mediated action as described by some scholars [2,25–27]. In this study, we observed significant increases in the protein levels of BMP-2 in MG-63 cells treated with both free SV and SV-loaded micelles. This is consistent with the previously published mechanism. In addition, the protein expression of BMP-2 in MG-63 cells treated with SV-loaded micelles with appropriate concentrations of SV was far higher than cells treated with an equivalent concentration of free SV. This is consistent with the ALP activity. All these results indicate that the enhanced differentiation and maturation of osteoblasts stimulated by SV-loaded PECL micelles may be associated with the increased expression of BMP-2 in osteoblasts, which is caused by the sustained release of SV.

Based on the cytologic results, we postulate that SV at appropriate concentrations could suppress the early proliferation of osteoblasts, promote osteoblast differentiation, and have a stimulatory effect on the expression of BMP-2, an osteoblast specific gene. Notably, SV-loaded micelles showed better biological efficacy, such as a reduction in osteoblast early proliferation inhibition, a promotion in osteoblast differentiation and mineralization, and the stimulation of BMP-2 gene expression. This improvement could be attributed to the sustained release of SV. It appears that the initial burst of suppression caused by SV-loaded PECL micelles reduced the osteoblast proliferation inhibition, whereas the prolonged release of SV enhanced the differentiation, mineralization and stimulatory effect on the expression of the BMP-2 gene.

Several problems with this study are worth noting. First, although the biocompatibility of the PECL copolymer has been previously demonstrated, the PECL micelles may possess low cytotoxicity because the PM displayed a significant reduction in their early proliferation rate compared to BK in the WST assay. The cytotoxicity of nanoparticles or micelles based on PECL copolymers has been examined in various cell types, such as MCF-7 cells [28], normal human fibroblast cells [29] and HepG2 cells [30], and all showed low cytotoxicity. Therefore, one may feel constrained to use micelles or nanoparticles based on PECL copolymers. However, the low cytotoxicity of PECL micelles with regard to MG-63 cells was exhibited only in the early phase of cell proliferation and not during the middle and late phases. This is most likely because the low cytotoxicity of PECL micelles may be related to the organic solvent residue, such as acetone, in the micelles, and would disappear once the residue volatilized. Investigations regarding the effect of dialysis time on cytotoxicity are needed for subsequent research. Second, there was no addition of an osteogenic induction medium, such as ascorbic acid, dexamethasone or β-glycerophosphate, prior to Alizarin Red S staining to avoid interference with the mineralization induction effect of the SV-loaded micelles. Appropriate levels of organic phosphate are usually required for mineralization to occur in culture [31] because osteoblast cultures grown in the presence of the alkaline phosphatase substrate, β-glycerophosphate, had mineral deposits. Our observation that MG-63 cells could produce mineral depositions, as visualized by Alizarin Red S staining after 7 days of culture without the addition of an osteogenic induction medium in culture, is an important finding. Furthermore, the mineralization assay in the absence of an osteogenic induction medium could faithfully represent the initial mineralization induction effect of SV-loaded micelles. Third, the mRNA expression levels of BMP-2 differed from the protein expression in cells grown with SV-loaded PECL micelles and free SV. Differences in mRNA expression were not considered significant for pairwise comparison among each concentration of SV in the SVPM and FSV groups and the PM and BK groups, and were dissimilar to the bar representing the mean shown in the statistical graph. The protein expression in the SVPM group at each concentration and in the FSV group at 2.5×10^{-7} and 2.5×10^{-8} M of SV were significantly higher than in the BK group. The occurrence of posttranslational modifications may explain this result. Furthermore, the protein level of BMP-2 has more functional relevance than the gene expression level. In the study by Ruiz-Gaspa et al. [23], a significant increase BMP-2 mRNA was observed in MG-63 cells cultures treated with only 10^{-7} M SV, which differed from this study. Error analysis in the experiments may explain this result. However, the results of both studies suggested a significant increase in the BMP-2 gene of MG-63 cells treated with SV. Fourth, qualitative analysis of the protein expression of other osteoblast-related genes, such as type I collagen, osteopontin, sialoprotein and osteocalcin, were not performed in this study. Ruiz-Gaspa et al. [23] demonstrated that SV enhanced the gene expression of collagen type 1 and osteocalcin in MG-63 cultures. Chen et al. [27] reported that SV promoted osteoblast differentiation by up-regulating the expression of BMP-2, ALP, sialoprotein, and type I collagen genes. In addition, two treatment groups with dissimilar results were worthy of further study. First, the results of both mineral deposition and ALP activity in the SVPM or FSV groups were dissimilar. Second, both the results of ALP activity and protein expression of BMP-2 were dissimilar. Therefore, the biological behavior of osteoblasts, such as proliferation, differentiation, and mineralization, could not be explained by BMP-2 gene expression only, and studies investigating the expression of other osteoblast-related genes are still required.

5. Conclusions

In this study, SV-loaded PECL micelles with sustained release profiles, have been developed and evaluated with osteoblast-like MG-63 cells *in vitro*. We have demonstrated that the SV-loaded micelles can enhance osteoblast differentiation and mineralization through BMP-2 mediated effects and suppression of the inhibition of osteoblast early proliferation. It can be concluded that the SV-loaded PECL micelles may be used clinically in the near future for bone loss diseases, including osteoporosis, because they have better bioavailability than free SV. However, more in-depth studies are still required to determine the exact mechanism of action of SV-loaded micelles for bone regeneration.

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