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Original Research

Combined delivery of PDGF-BB and BMP-6 for enhanced osteoblastic differentiation

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Abstract Natural microenvironment during bone tissue regeneration involves integration of multiple biological growth factors which regulate mitogenic activities and differentiation to induce bone repair. Among them platelet derived growth factor (PDGF-BB) and bone morphogenic protein-6 (BMP-6) are known to play a prominent role. The aim of this study was to investigate the benefits of combined delivery of PDGF-BB and BMP-6 on proliferation and osteoblastic differentiation of MC3T3-E1 pre-osteoblastic cells. PDGF-BB and BMP-6 were loaded in gelatin and poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) particles, respectively. The carrier particles were then loaded into 3D chitosan matrix fabricated by freeze drying. The fast release of PDGF-BB during 7 days was accompanied by slower and prolonged release of BMP-6. The premising release of mitogenic factor PDGF-BB resulted in an increased MC3T3-E1 cell population seeded on chitosan scaffolds. Osteogenic markers of RunX2, Col 1, OPN were higher on chitosan scaffolds loaded with growth factors either individually or in combination.

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However, OCN expression and bone mineral formation were prominent on chitosan scaffolds incorporating PDGF-BB and BMP-6 as a combination.

1 Introduction

Bone tissue engineering has emerged as a promising strategy for repairing bone defects by using supportive substitutes to enhance bone healing [1]. These substitutes are designed to promote bone matrix formation by mimicking the natural wound healing cues in bone and trigger the recruitment, proliferation and differentiation of bone progenitor cells [1, 2]. Within the complex cascade of this process biological growth factors are known to play a central role to accelerate the regeneration of defective bone tissue [2, 3]. In this respect, combining the delivery of biological growth factors and biomaterial scaffolds as a controlled environment for cell population has received considerable attention [1, 3].

Although there are a number of studies showing the use of individual growth factors encapsulated in scaffolds lead to successful generation of bone like constructs recent studies have focused on the importance and benefits of delivering multiple growth factors to provide biological cues in bone repair [4–9]. Osteoinductive bone morphogenetic proteins (BMPs) and mitogenic platelet-derived growth factor-BB (PDGF-BB) are reported as two of the most prominent factors for treatment of bone defects [10, 11]. PDGF-BB has been demonstrated to stimulate the proliferation and osteogenesis of diverse cell types as well as enhance bone formation in fracture and defect models [12]. Recombinant human PDGF-BB has been approved by US Food and Drug Administration (FDA) for use in

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diabetic foot ulcers due to its capacity in promoting wound healing [13, 14]. It has been reported that introduction of PDGF-BB results in the expansion of the number of proginetor cells available to induce bone repair [15, 16]. BMPs belong to the transforming growth factor-β (TGF-β) superfamily and reported to induce bone formation and osteoblastic differentiation [17]. Although most of the studies have been carried out on BMP-2 and -7 as they are approved regulators by FDA, several studies have shown that BMP-6 may be an even more potent osteogenic signal [18, 19].

The introduction of PDGF-BB as a mitogenic factor and BMP-6 as an osteogenic factor would offer an attractive strategy to mimic the bone healing cascade [2]. In this study, PDGF-BB was encapsulated in gelatin microparticles and BMP-6 was encapsulated in poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) sub-micron particles to acquire different release kinetics. Subsequently, particles carrying the growth factors are incorporated into biodegradable 3D chitosan matrix prepared by freeze-drying. Different biodegradation rates of particles induced an accelerated rate of release of PDGF-BB and slower rate of release of BMP-6. MC3T3-E1 preosteoblastic cells were seeded onto chitosan scaffolds containing either individual or combination of growth factors (Fig. 1). Proliferation and osteogenic differentiation were evaluated in vitro for each case.

2 Materials and methods

2.1 Materials

Chitosan derived from crab shell (Ch, deacetylation degree min. 85 %), poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) with 12 wt% PHV, polyvinyl alcohol (PVA) (average molecular wt. 13,000–23,000), dichloromethane (DCM), phosphate-buffered saline (PBS, pH: 7.4) tablets, tris HCL (10 mM, pH: 7.4), acidic gelatin (IEP: 5.0), glyoxal, olive oil and *n*-hexane were obtained from Sigma-Aldrich (Germany). Recombinant human bone morphogenic protein-6 (BMP-6) and enzyme-linked immonosorption assay (ELISA) kit were purchased from Cell Sciences (USA). Recombinant human platelet derived growth factor (PDGF-BB) and ELISA kit were obtained from RayBiotech (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hexamethyldisilazane (HMDS), fetal bovine serum (FBS), 1 % penicillin–streptomycin solution, ascorbic acid, α-MEM (with L-glutamine) and glycerol phosphate were obtained from Sigma-Aldrich (Germany). Glutaraldehyde (25 wt%) was purchased from Merck (Germany). Trizol was obtained from Invitrogen (Germany).

2.2 Preparation of BMP-6 loaded PHBV particles

BMP-6 loaded PHBV particles were prepared by using double emulsion-solvent diffusion method [20]. Briefly, 100 μL of an aqueous solution containing 20 μg BMP-6 (w1) was emulsified in PHBV solution (o1, 2 % (w/v) in DCM, 5 mL) by using a high-speed homogenizer (2500 rpm, 2 min). The first emulsion was then added into aqueous PVA solution (w2, 4 %, w/v, 10 mL) to form double emulsion (w1/o1/w2) and emulsified at 17,500 rpm for 15 min (Heidolph, Germany). Subsequently, w1/o/w2 emulsion was added to 50 ml of 3 mg/mL PVA solution. After stirring overnight, BMP-6 encapsulated PHBV particles (PHBV/BMP-6) were collected by centrifugation (10,000 rpm for 10 min), washed with Tris–HCL (pH: 7.4) and freeze-dried at −80 °C (Christ, Germany).
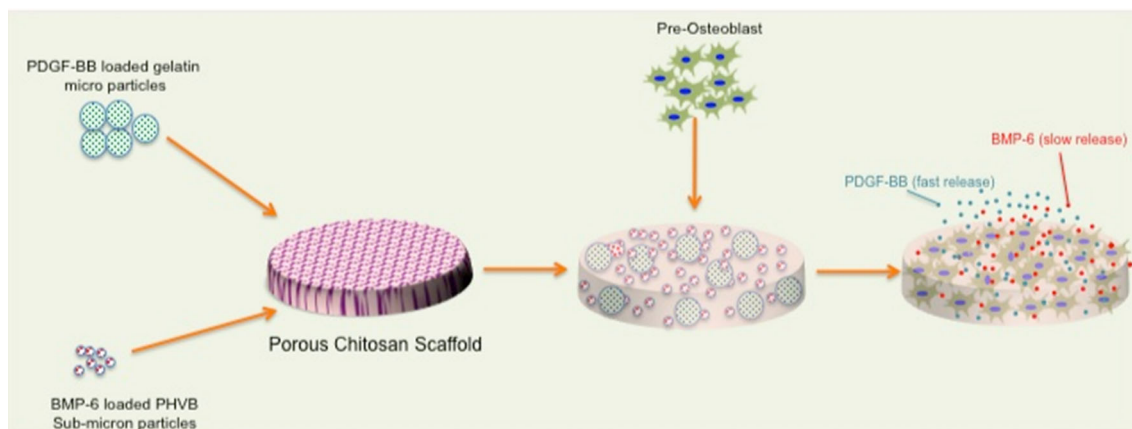

Fig. 1 Schematic diagram of the preparation process for dual growth factor encapsulated chitosan scaffolds to evaluate the proliferation and osteogenic differentiation of MC3T3-E1 pre-osteoblasts. Scaffold dimensions are 2 mm in thickness and 10 mm in diameter

2.3 Preparation of PDGF-BB loaded gelatin particles

Gelatin microparticles were prepared by using water-in-oil emulsion method [21]. Briefly, gelatin was dissolved in deionized water at 60 °C. The solution was added drop wise to olive oil while stirring at 500 rpm. The temperature of the emulsion was decreased to 15 °C under constant stirring for 30 min. Chilled acetone at 4 °C was added and gelatin microspheres were collected by filtration. After several washing steps with absolute ethanol and acetone, the microspheres were crosslinked in 20 mM glyoxal in aqueous ethanol for 15 h at room temperature. After washing with aqueous ethanol the microparticles were freeze-dried at −80 °C (Christ, Germany). PDGF-BB was loaded to the crosslinked microparticles by adsorption. Gelatin microparticles were vortexed with PDGF-BB solution (20 μL/mg particle, in PBS (pH: 7.4)) for 20 s and incubated at 4 °C overnight. After the incubation period PDGF-BB loaded microparticles (gelatin/PDGF-BB) were freeze-dried at −80 °C (Christ, Germany).

2.4 Preparation and characterization of chitosan scaffolds

Chitosan (Ch) scaffolds were prepared by freeze-drying method [22]. Ch solution of 2 % (w/v) dissolved in 0.2 M acetic acid was poured into 24-well tissue culture polystyrene dishes (TCPS) and frozen at −20 °C for 24 h. Porous scaffolds were obtained by lyophilization at −80 °C for 4 days. The lyophilized scaffolds were immersed in 96 % (v/v) ethanol for 24 h then in 70 % (v/v) ethanol for 1 h to stabilize the structure.

To prepare BMP-6 loaded chitosan scaffolds, PHBV/BMP-6 particles (2 mg PHBV particles/scaffold) were mixed with chitosan solution prior to freezing at −20 °C and lyophilization (Ch/BMP-6). For chitosan scaffolds incorporating PDGF-BB, gelatin/PDGF-BB microparticles were dispersed in PBS (1.5 mg microparticles in 300 μL PBS/scaffold) and loaded into Ch and Ch/BMP-6 porous scaffolds by suction (Ch/PDGF-BB and Ch/PDGF-BB + BMP-6). Eventually, 100 ng of PDGF-BB and/or 100 ng of BMP-6 were present within each scaffold.

Characterization studies were performed by using four groups of scaffolds:

- Group I: chitosan scaffolds (Ch)
- Group II: gelatin/PDGF-BB loaded Ch scaffolds (Ch/PDGF-BB)
- Group III: PHBV/BMP-6 loaded Ch scaffolds (Ch/BMP-6)
- Group IV: PHBV/BMP-6 and gelatin/PDGF-BB loaded Ch scaffolds (Ch/PDGF-BB + BMP-6)

The morphology of the scaffolds was observed using a scanning electron microscope (SEM, FEI Quanta 400F, USA). The samples were mounted on aluminum stubs, sputter coated with gold–palladium and examined under SEM at an accelerated voltage of 5–20 kV.

The pore size distribution of Ch, Ch/PDGF-BB, Ch/BMP-6 and Ch/PDGF-BB + BMP-6 scaffolds was examined by Mercury Intrusion Porosimetry (MIP, Quantachrome, USA). Mercury with a surface tension of 480 erg/cm^2 was used as the non-wetting liquid. Tests were performed under low pressure conditions in the range of 0–50 psi. The contact angle of mercury on scaffolds was 140°.

Swelling studies were performed to determine the water-uptake characteristics of the scaffolds. Dry samples were weighed (W_d) and placed in PBS (pH: 7.4) at 37 °C. The fully rehydrated samples were removed from PBS, blotted with filter paper and weighed again (W_s). Swelling ratios were determined gravimetrically by using Eq. 1.

$$Q = (W_s - W_d/W_d)/100 \tag{1}$$

In vitro release profiles of PDGF-BB and BMP-6 from chitosan scaffolds were examined for 21 days. Ch/PDGF-BB + BMP-6 scaffolds were placed in a container containing 2 mL PBS (pH: 7.4) with 0.1 % (w/v) sodium azide as a bacteriostatic agent and incubated at 37 °C with mild agitation (30 rpm). At designated time points 500 μL of the release medium were removed and replaced with the same amount of fresh PBS (pH: 7.4). The cumulative release amounts of PDGF-BB and BMP-6 from Ch/PDGF-BB + BMP-6 scaffolds were determined as a function of time by specific ELISA kits for each protein.

2.5 In vitro cell culture studies

2.5.1 Cell culture and seeding

Cell culture studies were carried out with MC3T3-E1 mouse pre-osteoblast cell line (doubling time: 36 h, No: RCB1126, Riken Cell Bank, Japan). The cells were subcultured in flasks using α-MEM supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin–streptomycin and maintained at 37 °C in a humidified 5 % CO_2 atmosphere (Heraus, Germany).

Ch, Ch/PDGF-BB, Ch/BMP-6 and Ch/PDGF-BB + BMP-6 scaffolds having 10 mm diameter and 2 mm thickness were sterilized with ethylene oxide for 240 min (Erna, Turkey). The sterilized scaffolds were individually placed into single wells of 24-well plate (Orange Scientific, Germany) and incubated in culture medium for 30 min before cell seeding. Thereafter, 50 μL of cell suspensions were injected into each scaffold (1×10^5 cells/scaffold) and were allowed to incubate (at 37 °C, 5 % CO_2) for 2 h.

Finally, 1 mL of culture medium was added to each well. At the first day of the culture, the media were replenished by osteogenic media (α-MEM supplemented with 10 % (v/v) FBS, 1 % (v/v) penicillin–streptomycin, 10 mM β-glycerol phosphate and 50 µg/mL ascorbic acid).

2.5.2 Cell viability

The viability of MC3T3-E1 cells was followed by MTT assay up to 21 days. At selected time intervals (1, 3, 5, 7, 14 and 21 days) the culture medium was aspirated and 600 µL of pre-warmed culture medium supplemented with 60 µL MTT solution (2.5 mg/mL MTT in PBS) was added to each well and incubated at 37 °C in a humidified 5 % CO_2 incubator for 3 h. After the incubation period the scaffolds were transferred into another 24-well plate. The formazan crystals were dissolved by adding 400 µL iso-propanol (in 0.04 M HCL). The optical density of the supernatant was measured spectrophotometrically at 570 nm with reference to 690 nm using a micro plate reader (Asys UVM 340, Austria).

2.5.3 SEM imaging

The morphology of MC3T3-E1 cells seeded on the scaffolds were observed by SEM (FEI Quanta, 400F, USA) at the 4, 7 and 14th days of culture period. The scaffolds were washed with PBS and the cells were fixed with 2.5 % (w/v) glutaraldehyde in 0.01 M PBS for 30 min at room temperature. Then, the samples were dehydrated in ethanol series (i.e., 30, 50, 70, 90 and 100 %) and rinsed with hexamethyldisilazane (HMDS) for 5 min. After complete drying, the scaffolds were mounted on aluminum stubs and gold–palladium prior to morphological observation under SEM.

2.5.4 Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR

The gene expressions of β-actin, RunX2, collagen type I (Col1), osteocalcin (OCN) and osteopontin (OPN) of the MC3T3-E1 pre-osteoblastic cells cultured on chitosan scaffolds were evaluated using RT-PCR (Light cycler Nano, Roche, Switzerland). At predetermined time intervals, (7, 14 and 21st days of culture) the medium was removed from scaffolds and the scaffolds were transferred to the RNase–free Eppendorf tubes. Then, they were broken into small pieces with a sterile needle. 500µL trizol reagent was added to each tube and vortexed for 30 s. The samples were incubated at 4 °C for 2 h. Ribonucleic acid (RNA) was then isolated from the samples at each time point using an RNeasy® Mini Kit (Qiagen, UK) by following the manufacturer's protocol. The purity and

concentration of the isolated RNA was determined by nanodrop (Thermoscientific 2000c, USA).

RT-PCR was performed in two steps by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and Hot FirePol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia). At the first step, cDNA were synthesized with reverse transcription at 25 °C for 10 min, at 40 °C for 120 min and at 85 °C for 5 min. At the second step, RT-PCR analysis was carried out with cDNA and forward-reverse primers. Following a RT-PCR initial activation step at 95 °C for 15 min, amplification was completed for 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. β-actin was used as housekeeping gene. Primers for each gene are: β-actin forward primer 5′-GTGCTATGTT GCCCTAGACTTCG-3′ Reverse primer 5′-GATGCCACA GGATTCCATACCC-3′ Col1 Forward primer 5′-CAAGA TGTGCCACTCTGACT-3′ Reverse primer 5′-TCTGACC TGTCTCCATGTTG-3′ OCN Forward primer 5′-CTTTC TGCTCACTCTGCTG -3′ Reverse primer 5′-TATTGCCC TCCTGCTTGG-3′ OPN Forward primer 5′-CACTTTCA CTCCAATCGTCCCTAC-3′ Reverse primer 5′-ACTCCT TAGACTCACCGCTCTTC-3′ RunX2 Forward primer 5′-GCATGGCCAAGAAGACATCC-3′ Reverse primer 5′-CCTCGGGTTTCCACGTCTC-3′

2.6 Statistical Analysis

All data were expressed as mean ± standard deviations of representative of three similar experiments carried out in triplicate. Statistical analyses were performed with Graphad Instat software. Student's t test was used to determine the differences among groups and P values less than 0.05 were determined significant.

3 Results

3.1 Characterization of scaffolds

Figure 2a–h shows the morphological images of Ch, Ch/BMP-6, Ch/PDGF-BB and CH/PDGF-BB + BMP-6 scaffolds observed by SEM.

Well-developed porous structure consisting of interconnected pores can be seen for all type of scaffolds. The pore size distribution in Ch scaffolds is between 30 and 150 µm (Table 1). The submicron sized BMP-6 loaded PHBV particles are thoroughly dispersed in the chitosan matrix structure (Fig. 2c, d) [20]. The interconnected pore structure is maintained in the presence of PHBV particles and the pore size distribution is measured as 40–200 µm for Ch/BMP-6 scaffolds (Table 1). Figure 2e and f show the microstructure of Ch scaffolds after embedding of

Fig. 2 SEM micrographs of scaffolds. **a** and **b** Ch, **c** and **d** Ch/BMP-6 **e** and **f** Ch/PDGF-BB and **g**, **h** Ch/PDGF-BB + BMP-6
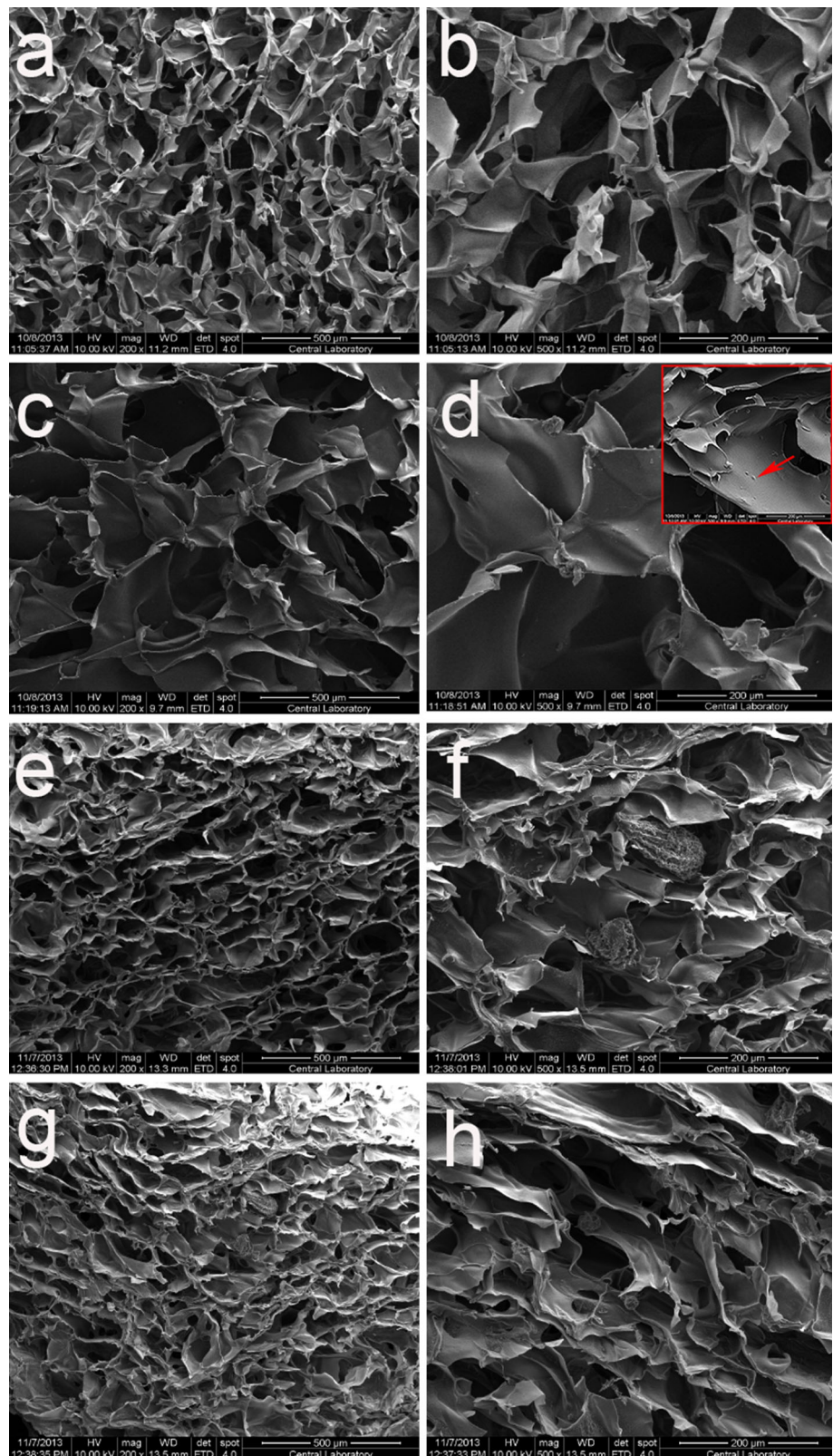
gelatin microparticles. The cross-linked gelatin microparticles retained their microspherical structure in the chitosan scaffold. Due to the larger size of the gelatin microparticles

(Supplementary data) the pore size distribution of the scaffolds is reduced to 10–50 μm (Table 1). As shown in Fig. 2g and h, similar characteristics are observed for Ch/

Table 1 Properties of Ch based scaffolds

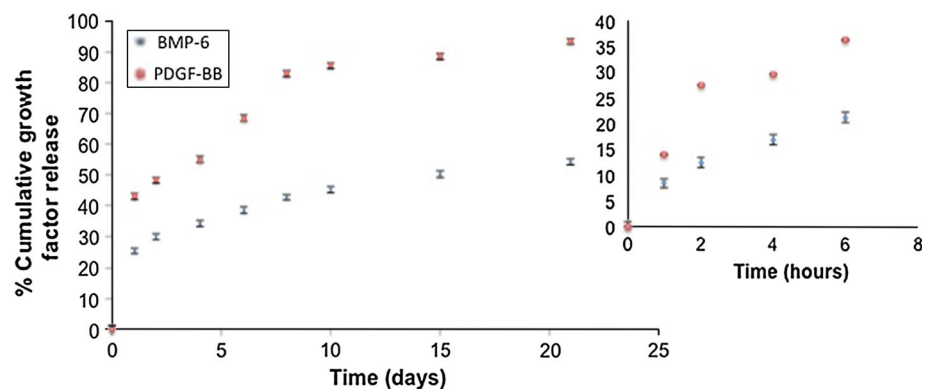| Scaffold	Ch	Ch/BMP-6	Ch/PDGF-BB	Ch/PDGF-BB + BMP-6
Pore size (μm)	30–150	40–200	10–50	5–50
Equilibrium swelling Q (%)	19.86 ± 1.70	24.22 ± 1.20	6.45 ± 0.26	6.41 ± 1.10

PDGF-BB + BMP-6 where the presence of gelatin microparticles causes a decrease in porosity. The pore size distribution for Ch/PDGF-BB + BMP-6 scaffolds was measured as 5–50 μm (Table 1).

The equilibrium swelling ratios show that the water up-take of Ch scaffolds change with the incorporation of protein loaded particles (Table 1). In the presence of PHBV particles, the swelling ratio is increased due to the increased stability of the pores. However, the micron sized gelatin particles embedded in the pore structure of Ch scaffolds cause an obstruction for water up-take both in Ch/PDGF-BB and Ch/PDGF-BB + BMP-6 scaffolds (Table 1).

The in vitro release behavior of PDGF-BB and BMP-6 from the delivery system was interpreted as the cumulative amount of growth factors over the time. Figure 3 shows the release behaviors of PDGF-BB and BMP-6 from Ch/PDGF-BB + BMP-6 scaffold for 21 days.

PDGF-BB release behavior shows an initial burst release of 43.0 ± 0.01 % within 1 day followed by a release profile reaching the plateau after 7 days. Nearly 90 % of PDGF-BB was released from the gelatin microparticles embedded in Ch scaffolds by 14 days. Release of BMP-6 encapsulated in PHBV particles also shows a similar burst release within 1 day but in a less amount of 25.4 ± 0.08 %. The release profile shows a moderate and sustained release between day 1 and 7 for BMP-6. At the end of 14 days BMP-6 release reached a cumulative of 50.2 ± 0.1 %. A comparison between the cumulative PDGF-BB and BMP-6 release profiles from Ch/PDGF-BB + BMP-6 scaffolds reveals that the release amount of PDGF-BB was higher than that of BMP-6. Moreover, release rate of BMP-6 was slower than that of PDGF-BB during the release period of 1–7 days.

3.2 In vitro MC3T3-E1 proliferation and osteoblastic differentiation

Cell culture studies were performed to investigate the proliferation, morphology and differentiation of pre-osteoblastic cells on Ch, Ch/PDGF-BB, Ch/BMP-6 and Ch/PDGF-BB + BMP-6 scaffolds. The scaffolds were 10 mm in diameter and 2 mm in thickness.

MC3T3-E1 pre-osteoblast proliferation results through 21 days of culture period determined by MTT assay are shown in Fig. 4.
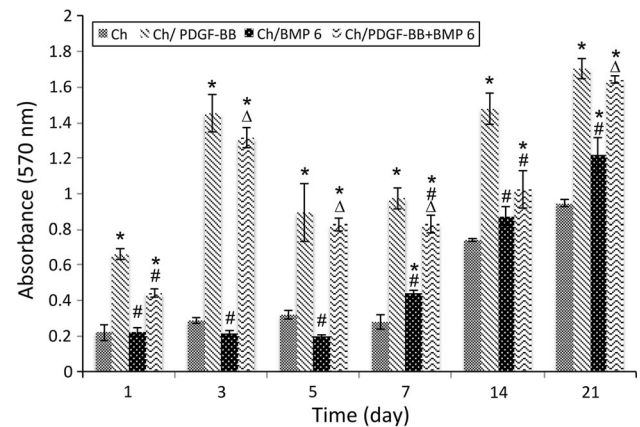

Fig. 4 MTT results of MC3T3-E1 cultured on chitosan scaffold (Ch), chitosan-PDGF-BB (Ch/PDGF-BB) scaffold, chitosan-BMP 6 (Ch/BMP 6) scaffold and chitosan-PDGF-BB + BMP 6 (Ch/PDGF-BB + BMP 6) scaffold (statistically significant differences n = 3, *$P < 0.05$, when control group is Ch scaffold scaffold; #$P < 0.05$ when control group is Ch/PDGF-BB scaffold; Δ$P < 0.05$ when control group is Ch/BMP 6 scaffold)

Fig. 3 In-vitro release profile of PDGF-BB (encapsulated in gelatin microparticles) and BMP-6 (encapsulated in sub-micron PHVB particles) from chitosan scaffold (Ch/PDGF-BB + BMP 6) during 21 days. The inserted graphic displays the release profile data for 8 h

The results demonstrate that all groups of scaffolds support the attachment and proliferation of MC3T3-E1 cells. However, Ch/PDGF-BB scaffolds are more conducive to proliferation compared to other groups. Cell proliferation on Ch and Ch/BMP-6 followed similar profiles during the first 4 days of culture period. No significant difference was observed on Ch and Ch/BMP-6 scaffolds in terms of cell proliferation. Starting from day 7 the cell density was higher on Ch/BMP-6 scaffolds when compared to Ch scaffolds. The Ch/PDGF-BB + BMP-6 scaffolds containing both growth factors displayed similar effect on cell proliferation with Ch/PDGF-BB scaffolds, with no significant difference on MTT results ($P > 0.05$).

Figure 5 illustrates the SEM images of MC3T3-E1 pre-osteoblasts cultured on chitosan based scaffolds at 7 and 14 days of culture. The cells on Ch scaffolds exhibited spherical morphology on day 7 and spreading behavior was not visible (Fig. 5a). No progress was detected on cell morphology as the culture period increased to 14 days. Mostly coagulated cells were sparsely distributed on the scaffold (Fig. 5b). Figure 5c and d reveal the increase in cell density on Ch/PDGF-BB scaffolds. Abundant cells adhered on Ch/PDGF-BB scaffolds and maintained lamellipodial extensions between the pores of the scaffold (insets in Fig. 5c, d). The morphology of MC3T3-E1 cells cultured on Ch/BMP-6 scaffolds are displayed in Fig. 5e and f. The cell density showed similarity to Ch scaffolds (Fig. 5a, b and e, f). However, the morphology of the adhered cells were different on Ch/BMP-6 scaffolds and cell spreading was clearly enhanced on day 14 in the presence of BMP-6 (Fig. 5f). Figure 5g and h illustrate MC3T3-E1 morphology on Ch/PDGF-BB + BMP-6 scaffolds. The cell density was similar to Ch/PDGF-BB and cell to cell connections displayed a network structure at 14 day culture (Fig. 5h). Formation of mineral deposits was observed on 7 days culture and detected by EDAX analysis (Fig. 6).

Real-time PCR results for RunX2, Col 1, OPN and OCN at days 7, 14 and 21 of culture are given in Fig. 7. The expression of early phase osteogenic marker RunX2 increased with time and elevated at 14 days. Higher expression was realized for Ch/PDGF-BB and Ch/PDGF-BB + BMP-6 (Fig. 7a). Expression of Col 1 peaked at 21 days and was significantly higher for protein loaded scaffolds (Fig. 7b). The expression of early stage marker OPN at 7 days was statistically higher on protein loaded scaffolds when compared to Ch. The expression levels of OPN continued to increase during the culture period and peaked at 21 days (Fig. 7c). OCN is an important marker in the late stage of the osteogenic differentiation cascade. Peak expressions were obtained at 21 days culture especially for BMP-6 loaded and PDGF-BB + BMP-6 loaded scaffolds (Fig. 7 d).

4 Discussion

Combined delivery of growth factors has been an attractive biomimetic strategy in regulation of effective bone tissue repair [7, 9, 23]. This study demonstrates that release of PDGF-BB and BMP-6 in combination enhances osteoblastic differentiation compared to single release of these growth factors. We prepared a chitosan scaffold incorporating PHBV/gelatin particle delivery system for the administration of two model proteins PDGF-BB and BMP-6. This work was first to evaluate the effect of PDGF-BB and BMP-6 combined delivery on osteoblastic differentiation in-vitro.

In this study, controlled delivery of PDGF-BB has been realized by loading the protein to gelatin microparticles. It has been reported that, the polyion complexation between gelatin and charged molecules of growth factors is an efficient method for minimal denaturation during the loading process [9, 21]. Here, PDGF-BB (isoelectric point (IEP) = 9.8) is well absorbed into the negatively charged gelatin microparticles by forming a polyionic complex. The glyoxal cross-linking enabled the decreased dissolution of gelatin for controlled release of PDGF-BB. The concentration of glyoxal was kept at 20 mM not to cause an adverse effect on cell proliferation. Kim et al. [9] have previously tested the cytotoxicity of glyoxal crosslinked gelatin microparticles and reported the non-cytotoxicity, especially for lower concentrations of glyoxal.

The controlled delivery of BMP-6 has been realized by loading the protein to PHBV submicron particles. The release of BMP-6 is controlled by the degradation of PHBV [20, 24]. PHBV particles have been reported as alternative carriers for delivery and sustained release of biological molecules due to their cost effective biotechnological production [20, 25]. In this study, the release rate of the growth factors is tailored by using gelatin and PHBV particles, which have different swelling and degradation characteristics [9, 20, 24]. Typically, the fast swelling and degradation of gelatin particles compared to PHBV caused faster elution of PDGF-BB than BMP-6. In addition, loading of PHBV/BMP-6 submicron particles during the scaffold preparation further contributes to sequester BMP-6 release where the particles are embedded in chitosan structure. On the other hand, gelatin microparticles carrying PDGF-BB are located inside the pores where chitosan structure does not cause an additional restriction for diffusion.

The release of specific growth factors, BMPs and PDGFs, is known to be critical for enhanced bone regeneration [26]. In natural cascade, the immediate release of PDGF-BB after injury initiates the signaling events for matrix deposition and induces migration of mesencyhmal

Fig. 5 SEM micrographs of MC3T3-E1 cells cultured on **a** and **b** chitosan scaffold (Ch), **c** and **d** chitosan-PDGF-BB (Ch/PDGF-BB) scaffold, **e** and **f** chitosan-BMP 6 (Ch/BMP 6) scaffold and **g** and **h** chitosan-PDGF-BB + BMP 6 (Ch/PDGF-BB + BMP 6) scaffold for 7 and 14 days
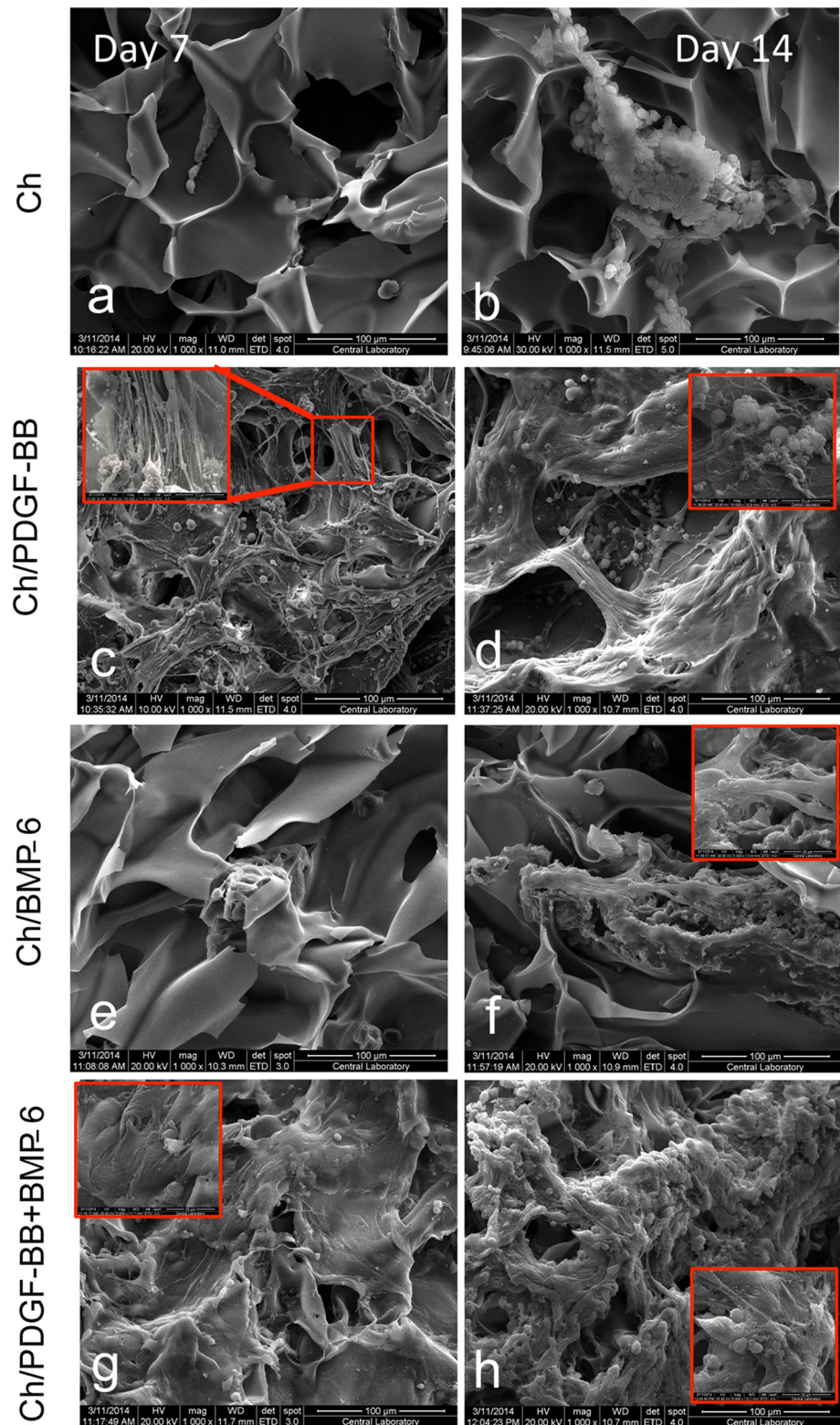
stem cells and osteoblasts [23]. After an initial expansion of these progenitor cells, BMPs act to accelerate the differentiation and mineral deposition [18, 23, 27]. We have demonstrated in this study, a fast and early release of PDGF-BB increases the attachment, spreading and proliferation of MC3T3-E1 preosteoblasts when loaded in chitosan scaffolds. When BMP-6 is used alone, MC3T3-E1 cell interaction in terms of attached cell density and
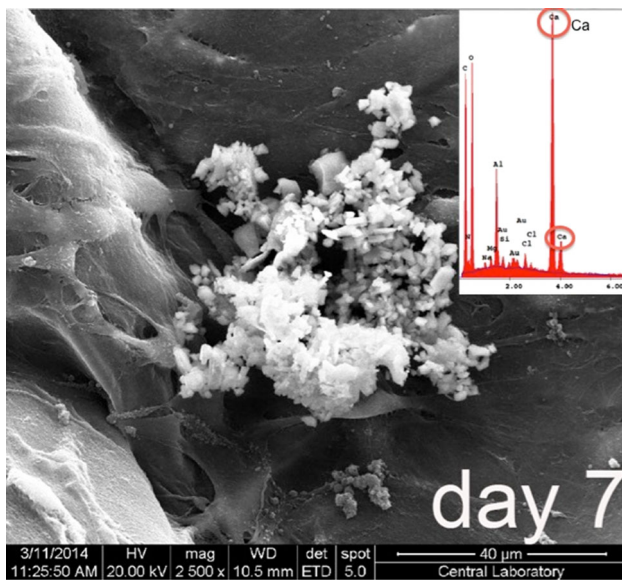
Fig. 6 SEM image and EDAX analysis of Ch/PDGF-BB + BMP-6 scaffold

morphological alteration remains limited in early period of the culture and increase in cell spreading is observed on day 14. Although BMP-6 loaded chitosan scaffolds show better performance than Ch scaffolds, cell spreading and proliferation on Ch/BMP-6 scaffolds are not triggered as much as on PDGF-BB loaded Ch scaffolds (Ch/PDGF-BB). These results reveal the importance of PDGF-BB as a mitogenic agent for increased cell interaction capacity.

Apart from its biocompatible characteristic, chitosan scaffold (Ch) without the incorporation of growth factors does not support MC3T3-E1 proliferation and differentiation. Initial cell interaction, which shall control the following differentiation steps is weak and should be supported by incorporation of attractant growth factors.

Expression of osteogenic markers is significantly different in growth factor loaded scaffolds. On chitosan scaffolds alone (Ch), preosteoblastic cell line (MC3T3-E1) shows limited intention for differentiation with an increase only in RunX2. Although BMP-6 is not as effective as PDGF-BB for increasing cell density, it plays a significant role in the differentiation process. The increase in gene expressions on Ch/BMP-6 scaffolds when compared to Ch alone predicts the enhancement of osteogenic differentiation of MC3T3-E1 preosteblasts. Meanwhile, the effect of PDGF-BB is pronounced as high levels of RunX2, Col1 and OPN expressions for Ch/PDGF-BB scaffolds. Zhao et al. [28] have previously reported that, PDGF-BB stimulates both the proliferation and differentiation of bone marrow stromal cells and plays an important role in bone formation. Anusaksathien et al. [29] has reported that PDGF-BB stimulates bone repair by expanding osteoblastic precursor cells. Our MTT results show the increased cell density on Ch/PDGF-BB scaffolds, which in turn leads to the increased osteogenic marker levels. However, expression of OCN indicating mature osteoblast differentiation and increase in bone mineral formation is significantly higher on scaffolds with dual growth factor, Ch/PDGF-

Fig. 7 Quantitative gene expression analysis by RT-PCR for **a** RunX2, **b** collagen type I (COL I), **c** osteopontin (OPN) and **d** osteocalcin (OCN) at the 7th, 14th and 21st days of culture. The y-axis represents the gene expressions normalized to β-actin. Data are expressed as mean value of triplicate samples and error bars as the standard deviation. Statistically significant differences are denoted by symbols; *$P < 0.05$, when control group is Ch scaffold scaffold; #$P < 0.05$ when control group is Ch/PDGF-BB scaffold; Δ$P < 0.05$ when control group is Ch/BMP 6 scaffold
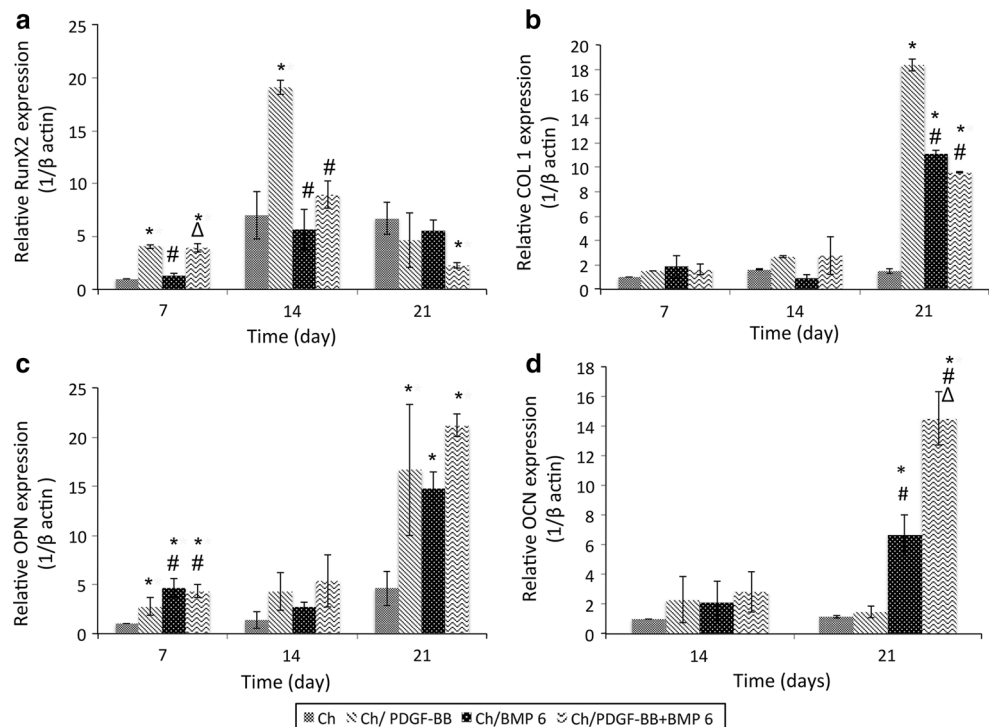
BB + BMP-6. The higher osteogenic capacity of Ch/PDGF-BB + BMP-6 scaffold is also evident from the detection of calcium mineral formation on day 7. Visser et al. have reported an increased calcium content and a synergic effect for BMP-6 when used together with an angiogenic molecule, basic fibroblast growth factor [18]. In this study, a larger population of cells due to the mitogenic capacity of PDGF-BB is affected by sustained release of BMP-6 promoting osteogenic differentiation. The dual delivery system designed in this study causes PDGF-BB to release quickly along with a slow sustained release of BMP-6 which provides an alternative to mimic the natural bone healing cascade.

5 Conclusion

In the present study, fibrous chitosan scaffolds incorporating PDGF-BB loaded gelatin microparticles and BMP-6 loaded PHBV submicron particles were used to investigate the effect of dual growth factor release on osteogenic differentiation. The results suggested that chitosan scaffolds that harness both the mitogenic PDGF-BB and osteogenic BMP-6 growth factors better induce osteogenic differentiation in vitro. Further in vivo study is in progress to evaluate the use of the designed system as a promising artificial bone substitute.

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