Sustained Delivery of BMP-2 and Platelet-rich Plasma-released Growth Factors Contributes to Osteogenesis of Human Adipose-derived Stem Cells

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abstract

Full article available online at Healio.com/Orthopedics. Search: 20120822-29

Platelet-rich plasma (PRP) has a pool of multiple growth factors efficient at inducing the proliferation and osteogenic differentiation of human adipose-derived stem cells (hADSCs). Bone morphogenetic protein (BMP)-2 is a strong stimulator for the osteogenic differentiation of hADSCs. The purpose of this study was to verify the effect of PRP-released growth factors and microsphere-encapsulated BMP-2 on the proliferation and osteoblastic differentiation of hADSCs and to construct a novel tissue-engineered bone. The BMP-2–loaded microspheres and hADSCs were embedded in activated PRP gel. Another 5 composites (hADSCs/platelet-poor plasma [PPP]; hADSCs/PRP; hADSCs/BMP-2/PPP; hADSCs/BMP-2/PRP; and hADSCs/BMP-2+microspheres/PPP) were also constructed. The DNA content, alkaline phosphatase activity, mRNA expression of alkaline phosphatase, osteopontin, osteocalcin, and mineralization of hADSCs in each composite were compared. The DNA content was higher in all PRP-containing composites, meaning that PRP-released growth factors stimulated proliferation of hADSCs. Alkaline phosphatase increased in BMP-2/PRP and BMP-2+microspheres/PRP composites in the first 7 days, meaning that BMP-2 had a synergistic effect with PRP in the early differentiation of hADSCs. Osteopontin, osteocalcin, and mineralization assays were higher in BMP-2+microspheres/PRP composite than in the BMP-2/PRP composite up to 21 days, meaning that a continuous delivery of BMP-2 stimulates osteoblastic differentiation of hADSCs at the early stage and the final maturation stage. These results suggest that sustained delivery of BMP-2 in combination with PRP is better than a single administration of PRP or BMP-2 in the osteogenic differentiation of hADSCs.

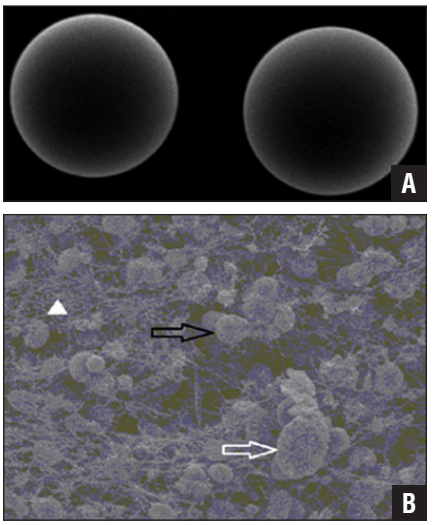

Figure: Scanning electron microscopic image (original magnification ×4.5 k) (A) and (original magnification ×2.0 k) (B) of microspheres (black arrow) and human adipose-derived stem cells (white arrow) distributed evenly in platelet-rich plasma gel. Triangle represents fibrin network of platelet-rich plasma clot.

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This study was funded by Wenzhou Science & Technology Bureau (2010S0555).

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doi: 10.3928/01477447-20120822-29

Bone defects, nonunion fractures, osteonecrosis, and osteoporosis challenge orthopedic surgeons. The use of stem cells as seed cells has given rise to a new method in treating these diseases. Among osteoregenerative cells, human adipose-derived stem cells (hADSCs) have shown the ability to differentiate into osteogenic lineages in vitro.[1] In addition, their harvest is convenient and their yield is approximately 40-fold higher than bone marrow mesenchymal stem cells.[2]

Platelet-rich plasma (PRP) is reportedly able to induce proliferation and osteogenic differentiation of hADSCs.[3] Bone morphogenetic protein (BMP)-2 is a formidable tool to induce osteogenic differentiation of hADSCs.[1] In vivo experiments have shown improved bone healing by PRP and its synergistic enhancement by BMP-2.[4-6] However, the synergistic effect of PRP and BMP-2 on the proliferation and osteogenic differentiation of hADSCs is still unclear.

Synergistic and sustained delivery of multiple growth factors may enhanced osteogenic differentiation of hADSCs to a higher degree than single administration.[7] In the current study, BMP-2 was encapsulated into microspheres and distributed equally in PRP gel with hADSCs. Human adipose-derived stem cells were used as seed cells, rapidly released growth factors from PRP and slowly released BMP-2 from microspheres were used as osteogenic agents, and fibrin reticulation was used as supporting scaffold.[8] The authors hypothesized that this biomaterial, which is characterized by a programmed delivery of growth factors, could stimulate the proliferation and osteogenic differentiation of hADSCs in vitro and osteogenesis in vivo.

MATERIALS AND METHODS

Isolation and Culture of hADSCs

Full ethical consent was obtained from all participants, and the study was granted ethical approval by the Medical Ethical Committee of the Wenzhou Medical College.

Human adipose-derived stem cells were isolated as previously described.[9] Adipose tissue was isolated from 5 healthy volunteers (3 men and 2 women; age range, 25-35 years) and enzymatically digested with 0.0075% type I collagenase. The remaining tissue was centrifuged for 10 minutes at 10,000 g, and the deposit was collected. The cell pellet was cultured in Dulbecco's Modified Eagle Media (DMEM), 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1% amphotericin in a humidified atmosphere of 5% CO_2 at 37°C. Stem cells were passaged every 3 to 4 days using 0.25% (w/v) trypsin solution, and cells obtained within 3 passages were used. To induce osteogenic differentiation, the culture medium was supplemented with 1028 M dexamethasone, 50 μg/mL ascorbic acid, and 5 mM β-glycerol phosphate.

Platelet-rich and Platelet-poor Plasma Preparation

Platelet-rich plasma was prepared by a 2-step centrifugation process.[10] Ten mL of blood withdrawn from the vein of each participant were initially centrifuged at 220 g for 15 minutes to separate the PRP and platelet-poor plasma (PPP) portions from the red blood cell fraction. The PRP and PPP portions were centrifuged again at 980 g for 10 minutes to separate the PRP from the PPP. The platelet count was adjusted to $1.5\pm0.1\times10^9$/mL in PRP and $8\pm0.5\times10^6$/mL in PPP. Platelet-rich plasma was activated and centrifuged at 3000 g for 10 minutes, and the supernatant was collected. The platelet-derived growth factor and transforming growth factor (TGF)-β1 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits as previously described.[3]

Preparation of BMP-2+Microspheres

Bone morphogenic protein-2–loaded microspheres were produced using water1/oil/water2 (W1/O/W2) double emulsion technology. The W1 solution was accomplished by introducing 50 μg BMP-2 and 5 mg bovine serum albumin into 50 μL polyethylene glycol solution (10%, v/v). The O dispersion was accomplished by introducing 200 mg of poly(lactide-co-glycolide) into 4 mL of organic solvent (dichloromethane/acetone=3/1 v/v). The primary water-in-oil dispersion was accomplished by introducing O dispersion into W1 dispersion and emulsifying by sonication (40 W for 30 seconds). The water-in-oil dispersion was then introduced into W2 dispersion containing 2% polyvinyl alcohol and 2% NaCl and stirred at 0°C for 5 minutes to create the double emulsion. Finally, the W1/O/W2 solution was introduced into 5% NaCl solution and stirred for 5 hours to evaporate the organic solvents. The microspheres were collected by high-speed centrifugation, repeated washing, and lyophilization.

Construction of 3-dimensional Composite

Poly(lactide-co-glycolide) cube scaffold ($5\times5\times4$ mm^3; 100 to 300 μm in pores; porosity >95%) was sterilized with 75% ethanol for 12 hours, followed by 2 saline washes and a Dulbecco's Modified Eagle Medium wash for 30 minutes before use. After incubation at 37°C for 6 hours to evaporate most of the water, the cubes or disks could be used.

Six composites were constructed for the in vitro study: composite I, ADSCs/PPP; composite II, ADSCs/PRP; composite III, hADSCs/BMP-2/PPP; composite IV, hADSCs/BMP-2/PRP; composite V, hADSCs/BMP-2+microspheres/PPP; composite VI, hADSCs/BMP-2+microspheres/PRP.

Human adipose-derived stem cells were suspended in PPP or PRP at a density of 5×10^6/mL, 100 ng/mL of BMP-2, and 2.5 mg of microspheres. An aliquot of 90-μL composite suspension was dropped onto poly(lactide-co-glycolide) cubes, followed by 10 μL of activating solution (10,000 units of bovine thrombin dissolved in 1 mL of 10% $CaCl_2$). Thirty minutes after the reaction, the composites were cultured in 5 mL culture medium.

Measurement of Size Distribution

The dried microspheres were redispersed in distilled water and assessed by laser light scattering. Microspheres size distribution was expressed as mean diameter and coefficient variation value.

Morphology of Microspheres and Composite

Surface morphology of microspheres and composite scaffold were measured by scanning electron microscopy (S-800; Hitachi High-Technologies Corp, Tokyo, Japan). The specimens were put on metal stubs and coated with a gold film approximately 60 nm thick under a reduced pressure with a JFC-1600 fine coater (JEOL Ltd, Tokyo, Japan).

Table

Real-time Polymerase Chain Reaction Primers Sequences for Markers of Osteoblastic Differentiation

Target Gene	Sequence (5′ to 3′)
Osteocalcin	Forward GTG CAG AGT CCA GCA AAG GT
	Reverse TCA GCC AAC TCG TCA CAG TC
Osteopontin	Forward CTC AGG CCA GTT GCA GCC
	Reverse CAA AAG CAA ATC ACT GCA ATT CTC
ALP	Forward CGC TGT GTC AAC TCC ACC T
	Reverse CCA GAA GGT TCT GTT AAC HG
GAPDH	Forward CCT CAA GAT CAT CAG CAA T
	Reverse CCA TCC ACA GTC TTC TGG GT

Abbreviations: ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenasen.

Encapsulation Efficiency Evaluation

Microspheres weighing 10 mg were rinsed with acetonitrile 3 times. After centrifuging (16,000 rounds/min) for 10 minutes, the precipitation was dissolved by isotonic phosphate-buffered saline.

Encapsulation efficiency (%)=(total amount of BMP-2 loaded/total amount of BMP-2)×100.

BMP-2 Release Studies

Release was determined by incubating microspheres and composite scaffold in 4 mL phosphate-buffered saline (pH 7.4) with 0.02% sodium azide at 37°C. The tubes were centrifuged at 5 and 12 hours and every other day starting on day 1 and ending on day 29. The collected 0.8 mL supernatant was assayed by ELISA for BMP-2 concentration, and 0.8 mL of additional phosphate-buffered saline was added to the remaining microspheres for continued incubation.

Cell Proliferation by Analysis of DNA Content

At 1, 4, 7, and 14 days of culture, scaffolds were harvested, cut into pieces, and lysed via lysis buffer (50 mM Tris-buffered saline, 1 mM EDTA, and 1 mM 2-mercaptoethanol). The lysate was sonicated and centrifuged (5 min, 12,000 g, 4°C). A portion of the collected supernatant was combined with Hoescht 33258 solution. The amount of DNA was measured with a fluorometer and standard solutions of calf thymus DNA.[11] This test was repeated 3 times.

Alkaline Phosphatase Assay

At 1, 7, 14, and 21 days of culture, alkaline phosphatase activity assay was performed as previously described.[11] One hundred µL of the supernatant were collected and mixed with 400 µL of p-nitrophenyl phosphate as substrate and incubated at 37°C for 15 minutes. The reaction was terminated by adding 50 µL NaOH. Absorbance was read on a spectrophotometer at 405 nm. The total protein contents were determined with the bicinchoninic acid method. This test was repeated 3 times.

Calcium Content of the Cultures

On day 21, scaffolds in each group were collected and washed with 0.05 M Tris-buffered saline, cut into small pieces, and incubated in 500 µL of 0.5 M HCl for 18 hours. After centrifugation, the supernatant was collected. The calcium content in the supernatant was measured with a commercially available kit. This test was repeated 3 times.

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction was used to detect the expression of several osteogenic differentiation-related marker genes (alkaline phosphatase, osteocalcin, and osteopontin) at 1, 5, 9, and 14 days of culture. The total RNA per composite was extracted using TRIzol (Invitrogen, Carlsbad, California). First-strand complementary DNAs (cDNAs) were reverse-transcribed from total RNA of each sample by oligo(dT) primer using the DyNAmo cDNA synthesis kit (Thermo Fisher Scientific Inc, Waltam, Massachusetts). Real-time polymerase chain reaction using the iCycler system (Bio-Rad Laboratories, Hercules, California) was performed with the single-stranded cDNA sample using SYBR Green Master mix (Invitrogen). The mRNA expression level of the target gene in each treatment group was determined by normalizing its expression level to that of the glyceraldehyde-3-phosphate dehydrogenase gene, which was used as an internal control. Primers of tested genes are shown in the Table.

Statistical Analysis

All quantitative data were expressed as mean±SD and analyzed with SPSS version 17.0 software (SPSS Inc, Chicago. Illinois). Equality of variances was verified using the Bartlett test. Data differences among each group were compared using the Student-Newman-Keuls q test. Statistical significance was set at $P<.05$.

RESULTS

Microspheres and 3-dimensional Composite Scaffold Characterization

All microspheres had a spherical shape with a smooth surface. The microspheres did not stick together (Figure 1A). The composite had good dispersity (ie, microspheres and hADSCs distributed evenly in PRP or PPP) (Figure 1B). The mean diameter of microspheres was 9.86 μm (Figure 1C). Encapsulation efficiency of BMP-2 in microspheres was 76%.

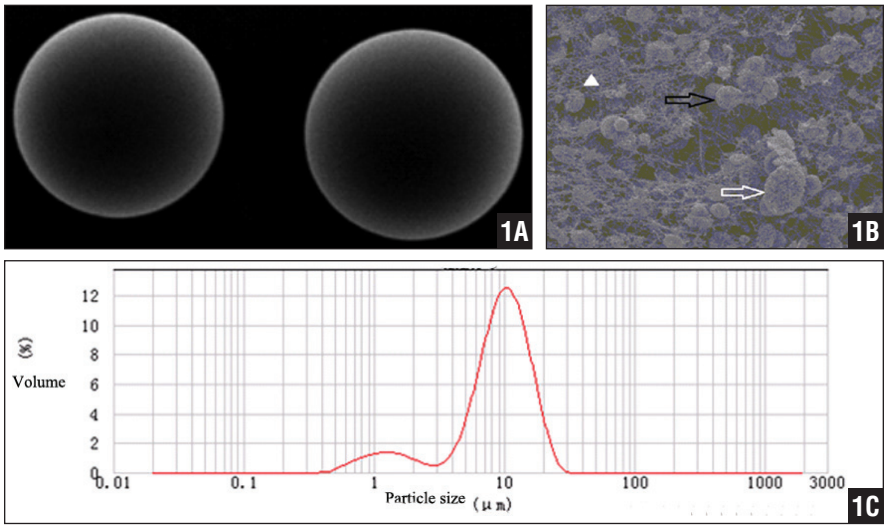

Figure 1: Scanning electron microscopic image (original magnification ×4.5 k) (A) and (original magnification ×2.0 k) (B) of microspheres (black arrow) and human adipose-derived stem cells (white arrow) distributed evenly in platelet-rich plasma gel. Triangle represents fibrin network of platelet-rich plasma clot. The mean diameter of microspheres was 9.86 μm (C).

Cumulative BMP-2 Release in Microspheres and Composite Scaffold

The cumulative release of BMP-2 absorbed in scaffolds III and IV or scaffolds V and VI is shown in Figures 2A and 2B, respectively. Simple adsorption of BMP-2 in PRP hydrogel was not efficient because nearly 90% of the BMP-2 was released in 5 days (Figure 2A). In contrast, when growth factor was loaded in microspheres, significantly lower burst release values were obtained (Figure 2B). The release rates of BMP-2 by the PRP exhibited similar profiles to that in the PPP.

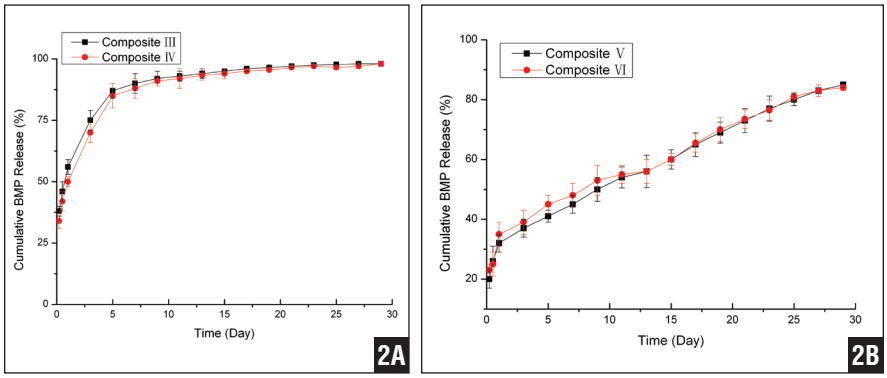

Figure 2: Graph of cumulative bone morphogenic protein (BMP)-2 release of BMP-2 composites showing that approximately 90% of the BMP-2 was released in 5 days (A). Graph of cumulative BMP-2 release of BMP-2+microspheres composities showing that a significantly lower burst release was obtained (B).

Platelet Counts and Levels of Growth Factors

The platelet counts were adjusted to $1.2\pm0.1\times10^9$/mL in PRP and $8\pm0.5\times10^6$/mL in PPP. The content of TGF-β1 in PRP was 450.5±21.4 ng/mL. The content of platelet-derived growth factor-AB in PRP was 85.8±13.2 ng/mL.

hADSCs Proliferation in Composites

Human adipose-derived stem cells showed a significantly higher increase in proliferation rate in PRP-containing composites than in PPP-containing composites up to day 14 ($P<.05$). The growth factors in PRP increased hADSCs proliferation significantly more in composites II, IV, and VI than in the control group (PPP) (composite I), which contained no growth factors. No significant differences were found between composites III and V and the control group ($P>.05$). The combination of BMP-2 with PRP increased hADSCs' proliferation more than did BMP-2+PPP (composite III) but produced no increase in proliferation compared with PRP alone (composite II) ($P>.05$). The combination of BMP-2+microspheres with PRP (composite VI) seemed to play the same role in hADSCs' proliferation as did the combination of BMP-2 with PRP (composite IV) ($P>0.05$). The amount of hADSCs in the BMP-2±microspheres composite was higher than that in BMP-2 composite, but the difference was not significant ($P>.05$) (Figure 3).

Osteogenic Differentiation of hADSCs in 3-dimensional Composites

In the early culture period, alkaline phosphatase activity in composites IV and VI showed the fastest increase and

reached the highest level of all composites on day 14 ($P>.001$). In the late culture period, alkaline phosphatase activity began to decrease in all composites, with the highest level in composites IV and VI ($P>.01$) (Figure 4A). Signifcant differences were also found between BMP-2/PPP (composite III) and PPP (composite I) ($P<.05$) and between BMP-2 microsphere/PPP (composite IV) and PPP (composite I) ($P<.05$). A similar change occurred in the mRNA expression of alkaline phosphatase (Figure 4B).

Significant increases were noted in osteopontin concentrations in composites II to VI during a 7-day culture period ($P>.05$). The BMP-2/PRP enhanced osteopontin expression to a higher level than did a single administration of BMP-2 and PRP ($P>.01$). The BMP-2/PPP had a similar effect on hADSC ($P>.05$). The osteopontin mRNA expression in composites V and VI increased significantly on day 14 ($P<.01$) and reached the highest level of all composites on day 21 ($P<.001$) (Figure 4C). Similar changes were also noted in osteocalcin mRNA expression. However, the osteocalcin mRNA expression in composite V did not exceed that in composite IV on day 14 and reached the highest level on day 21 ($P<.001$) (Figure 4D). The highest calcium content was found in composites V and VI ($P<.001$). Calcium content was higher in the BMP-2/PRP composites than in the PRP or BMP-2/PPP composites ($P<.01$). A single administration of PRP (composite II) and BMP-2 (composite III) stimulated a higher degree of calcium content than did PPP, but the difference was not significant ($P<.05$) (Figure 5).

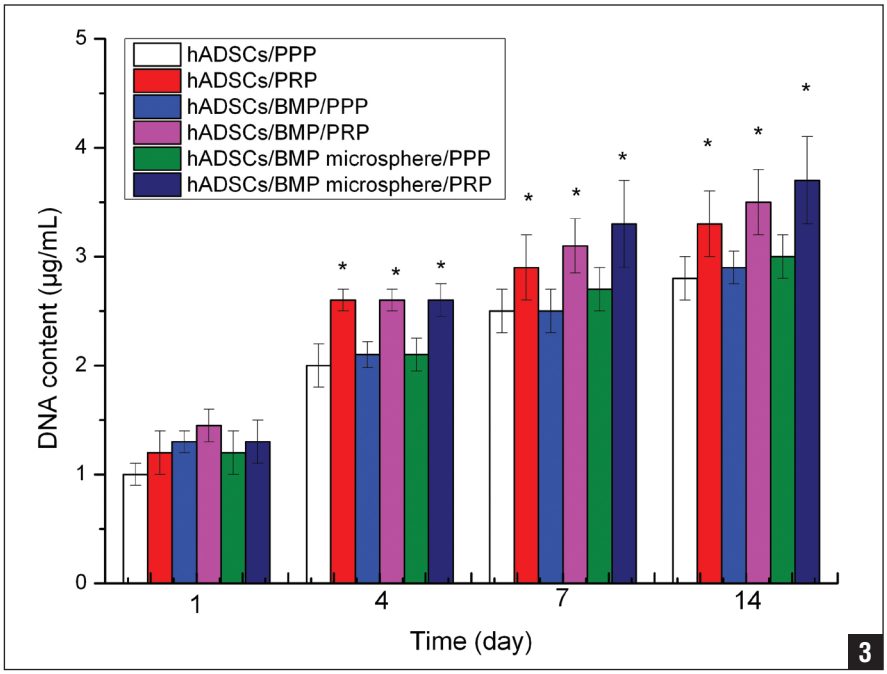

Figure 3: Graph showing the proliferation of human adipose-derived stem cells (hADSCs) at days 1, 4, 7 and 14. A significantly higher increase in proliferation rate occurred in the platelet-rich plasma (PRP) composite than in the platelet-poor plasma (PPP) composite up to day 14 ($P<.05$). No significant differences were found between composites III and V and the control group (composite I) ($P>.05$). The combination of bone morphogenetic protein (BMP)-2 with PRP increased hADSCs' proliferation more than did BMP-2 and PPP but produced no increase in proliferation compared with PRP alone ($P>.05$). The combination of BMP-2+microspheres with PRP seemed to play the same role in hADSCs' proliferation as did the combination of BMP-2 with PRP ($P>.05$). Asterisk indicates $P<.05$.

Discussion

It has been suggested that specific growth factors and cytokines may regulate fracture healing during the different stages. Growth factors released sustainably in a manner that mimics the physiological process of fracture healing in vivo enhanced tissue regeneration to a higher degree than did a single administration.[12-16]

Platelet-rich plasma is a pool of 30 autologous growth factors reported to induce cell proliferation and osteogenic differentiation of hADSCs.[3] When activated, a 7-fold increase of TGF-β1, 30-fold increase of platelet-derived growth factor, and 10-fold increase of vascular endothelial growth factor could be seen in PRP compared with whole blood.[17] Platelet-derived growth factor and insulin-like growth factor-1 in combination are efficient in driving osteogenesis of hADSCs.[18] Transforming growth factor-β1 stimulates osteogenesis of hADSCs.[19] However, the secretion of these growth factors begins within 10 minutes after clotting. More than 95% of the growth factors are secreted within 1 hour, followed by secretion of the remaining growth factors for an additional 7 days.[20] This short-term effect is not efficient for bone healing in vivo.

Bone morphogenic protein-2 was expressed during all stages of healing, and the application of bone morphogenic protein as an osteoinductive agent is superior compared with that of PRP.[21,22] Simultaneous delivery of PRP combined with BMP-2 enhances bone healing significantly in vivo.[4,6] To mimic an in vivo bone-healing process, sustained delivery of BMP-2 combined with PRP is used to induce osteogenic differentiation of hADSCs. A cocktail of growth factors released from activated platelets are predominantly expressed during the early phase and collaborate with BMP-2 to stimulate proliferation and early osteoblastic differentiation of hADSCs, whereas BMP-2 is continuously released from microspheres to induce further osteogenesis of hADSCs and mineralization. In the current study, proliferation of hADSCs was mainly in-

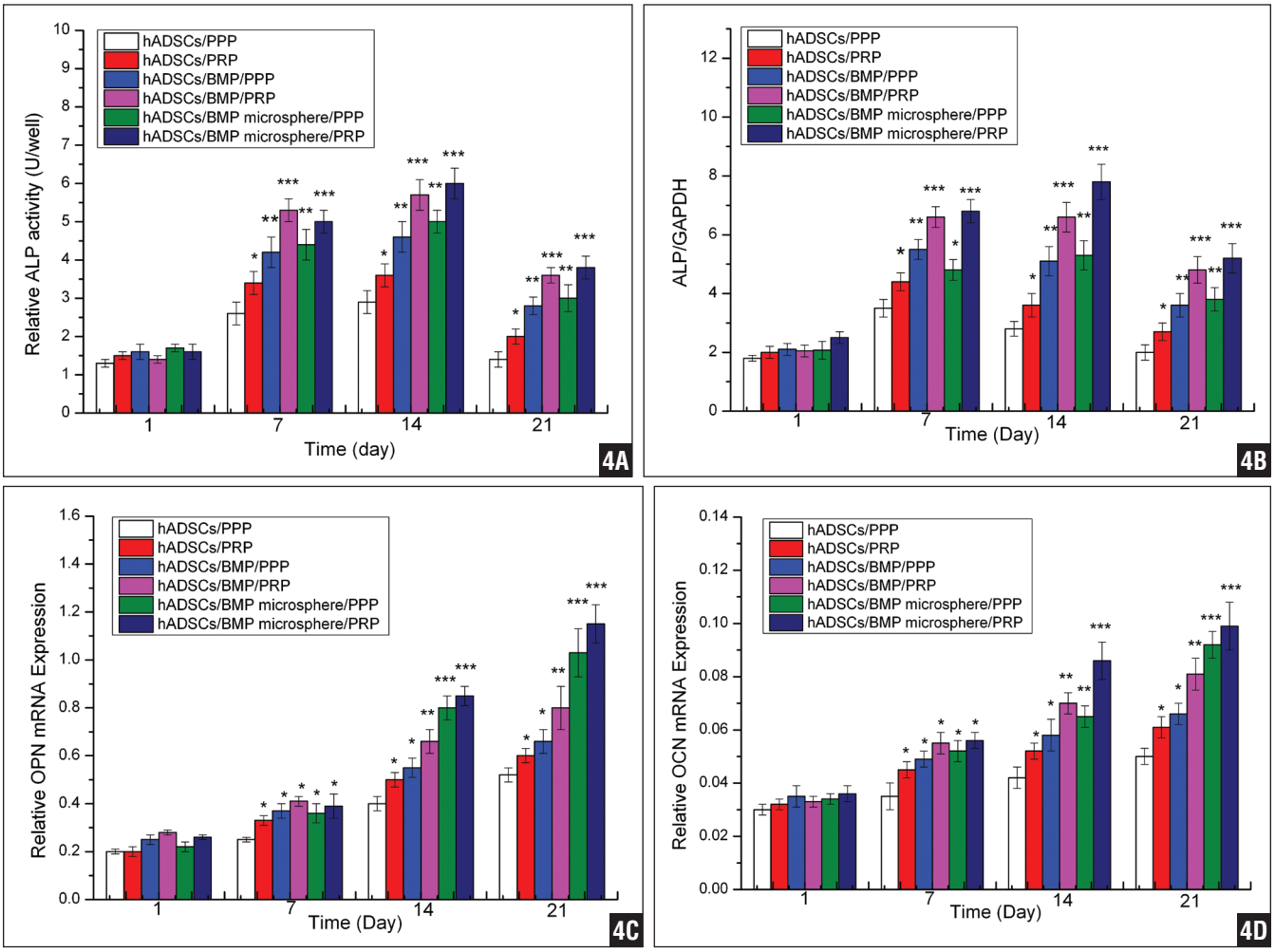

Figure 4: Graph showing that in the early culture period, alkaline phosphatase (ALP) activity in composites IV and VI showed the fastest increase and reached the highest level of all groups on day 14 compared with composite I (***$P<.001$). In the late culture period, alkaline phosphatase activity began to decrease in all groups, with the highest levels in composites IV and VI compared with composite I (**$P<.01$) (A). Graph showing the same change in the expression of alkaline phosphatase (ALP) mRNA (B). Graph showing that significant increases were noted in osteopontin (OPN) concentrations in composites II through VI during a 7-day culture period compared with composite I (*$P<.05$). Bone morphogenetic protein (BMP)-2 combined with platelet-rich plasma (PRP) (composites IV and VI) enhanced osteopontin expression to a higher level than did a single administration of PRP (composite II) and BMP-2 (composites III and V) (**$P<.01$). Osteopontin mRNA expression in composites V and VI increased significantly on day 14 compared with composite I (**$P<.01$) and reached the highest level of all groups on day 21 compared with composite I (***$P<.001$) (C). Graph showing similar changes noted in osteocalcin (OCN) mRNA expression (D). Abbreviations: hADSCs, human adipose-derived stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPP, platelet-poor plasma.

fluenced by PRP-released growth factors because all composites supplemented with PRP showed a significantly higher DNA content than that in PPP composites. Regarding osteogenic differentiation, the activity and mRNA expression of the early stage marker (alkaline phosphatase) increased in BMP-2/PRP and in BMP-2+microspheres/PRP in the first 7 days means that BMP-2 has a synergistic effect with PRP in the early differentiation of hADSCs. In addition, BMP-2 showed a higher alkaline phosphatase activity than did PRP. The mRNA expression of the late stage marker (osteocalcin and osteopontin) and mineralization assays were higher in the BMP-2+microspheres/PRP composite than in the BMP-2/PRP composite up to 21 days. This result means that a continuous delivery of BMP-2 stimulates osteoblastic differentiation of hADSCs in the early stage and the final maturation stage.

Platelet-rich plasma–released growth factors are conducive to inducing osteogenesis, and the 3-dimensional fibrin scaffold can be applied as a carrier for growth factors and hADSCs.[8] In addition, the hydrogelling structure provides better support and even distribution of BMP-2–loaded microspheres. Scanning electron

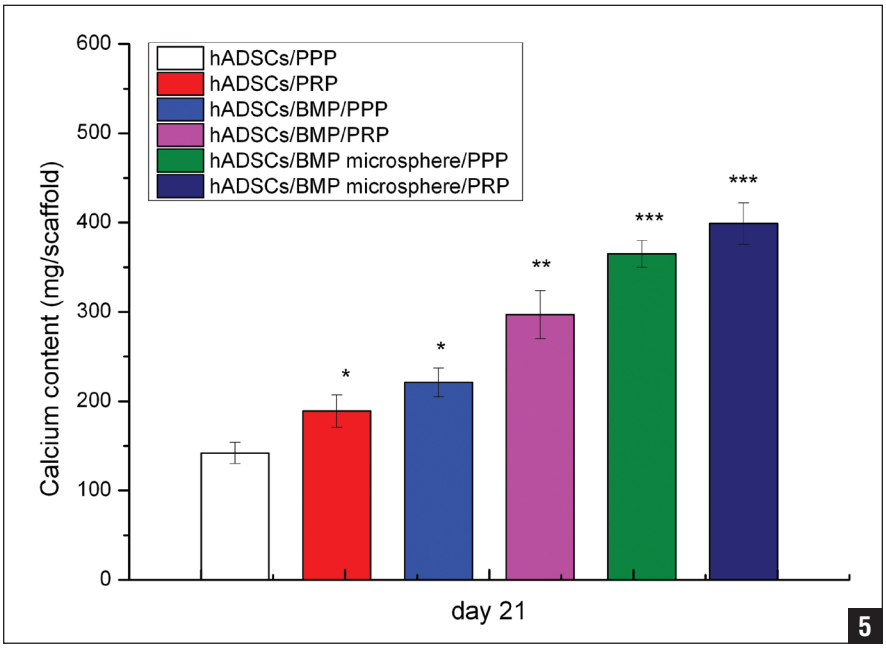

Figure 5: Graph showing calcium content in the 3-dimensional cell culture on day 21. The highest calcium content was found in composites V and VI compared with composite I (***P<.001). Calcium content was higher in the bone morphogenetic protein (BMP)-2/platelet-rich plasma (PRP) composite (IV) (**P<.01) than in the PRP composite (II) (*P<.05) or BMP-2 composite (III) (*P<.05). Abbreviations: hADSCs, human adipose-derived stem cells; PPP, platelet-poor plasma.

microscope examination found a highly porous structure of PRP, which is similar to the extracellular matrix. Human adipose-derived stem cells and microspheres were enwrapped by fibrin. The fibrin could maintain the 3-D network space and provide a matrix for hADSCs' adherence and proliferation. The insoluble fibrin networks and upregulating collagen I might effectively promote synthesis of extracellular matrix and osteogenic differentiation of hADSCs. Sustained delivery of BMP-2 combined with PRP to a target site will provide orthopedic surgeons with a useful tool for the reconstruction of bone defects or nonunion.

The lack of in vivo experiments is the main limitation of this study. The in vitro data cannot always mimic in vivo conditions, so the results of the study need further validation in future. In addition, the calcium content measurement may not be able to reflect the true degree of mineralization in the culture system.

Conclusion

Synergistic delivery of PRP-released growth factors and BMP-2 effectively stimulates the proliferation and early osteogenic differentiation of hADSCs. Sustained delivery of BMP-2 combined with PRP stimulates osteoblastic differentiation of hADSCs at the early stage and the final maturation stage. Due to the hydrogelling properties of the PRP clot, platelet-rich gel may be an ideal carrier to support hADSCs and BMP-2+microspheres and be an ideal tissue-engineering material for bone healing.

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