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Effects of Culture Conditions on Osteogenic Differentiation in Human Mesenchymal Stem Cells

SONG, SU JIN[1], OJU JEON[1], HEE SEOK YANG[2], DONG KEUN HAN[3], AND BYUNG-SOO KIM[2]*

[1]*Department of Chemical Engineering, and* [2]*Department of Bioengineering, Hanyang University, Seoul 133-791, Korea*
[3]*Biomaterials Research Center, Korea Institute of Science and Technology, Seoul 130-650, Korea*

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Abstract Human bone marrow-derived mesenchymal stem cells (hBMMSCs) must differentiate into osteogenic cells to allow for successful bone regeneration. In this study, we investigated the effects of different combinations of three soluble osteogenic differentiation-inducing factors [L-ascorbic acid (AC), β-glycerophosphate (βG), and bone morphogenic protein-2 (BMP-2)] and the presence of a hydroxyapatite (HA) substrate on hBMMSC osteogenic differentiation *in vitro*. hBMMSCs were cultured in medium containing various combinations of the soluble factors on culture plates with or without HA coating. After 7 days of culture, alkaline phosphatase (ALP) activity, calcium deposition, and osteoprotegerin (OPG) and osteopontin (OPN) expression were measured. The effects of individual and combined factors were evaluated using a factorial analysis method. BMP-2 predominantly affected expression of early markers of osteogenic differentiation (ALP and OPG). HA had the highest positive effect on OPN expression and calcium deposition. The interaction between AC, βG, and HA had the second highest positive effect on ALP activity.

Keywords: Bone, differentiation, mesenchymal stem cell

Human bone marrow-derived mesenchymal stem cells (hBMMSCs) possess an osteogenic capacity that can be used for bone regeneration [18, 31]. Repairing adult bone fractures involves bone marrow-derived mesenchymal stem cells (BMMSCs) invading the fracture site, proliferating, and differentiating into bone. These mesenchymal progenitors (also known as mesenchymal stem cells or marrow stromal cells) can be isolated from bone marrow and cultured under conditions that promote differentiation into specific mesenchymal phenotypes, such as osteoblasts. A study suggested that transplanting hBMMSCs that have been differentiated into a bone cell lineage *in vitro* would be better than transplanting undifferentiated hBMMSCs for *in vivo* bone regeneration [27]. In recent years, the hBMMSC osteogenic differentiation procedure has been extensively studied. Several researchers have reported methods to enhance BMMSC osteogenic differentiation by modifying the culture conditions with medium additives [3, 4, 9, 18, 20, 23, 24, 28, 30, 37], perfusion culture [11, 33, 42], mechanical stress [16, 26, 35], and scaffolds [14, 17, 19, 26, 40].

hBMMSC differentiation into osteoblasts can be induced by adhesives, such as hydroxyapatite (HA) [23], and soluble factors, such as dexamethasone [3, 18], L-ascorbic acid (AC) [9, 18], β-glycerophosphate (βG) [9, 18], TGF-β [4, 20], and bone morphogenic protein [20, 24, 28, 30, 37]. Previous studies have shown that dexamethasone, a synthetic glucocorticoid, can induce human bone marrow stromal cells to take on an osteoblastic phenotype [6], but dexamethasone showed the cytotoxicity in cell culture [15]. AC is also necessary for osteogenic collagen production and enhances alkaline phosphate (ALP) activity [7]. AC and βG are required for forming and mineralizing the extracellular matrix in bone marrow stromal cell culture [25]. No study has compared the effects of these osteogenic factors and interactions between the factors on *in vitro* hBMMSC osteogenic differentiation.

In this study, hBMMSCs were cultured in medium including various combinations of soluble factors on culture plates with or without an HA coating. After 7 days of culture, alkaline phosphatase (ALP) activity, calcium deposition, and osteoprotegerin (OPG) and osteopontin (OPN) expression were measured. The effects of each factor alone and the interactions between the factors were evaluated using a factorial analysis method.

Materials and Methods

Coating Culture Substrate with HA

Culture plates were HA coated by plasma treating of the culture surface and incubated in simulated body fluid

*Corresponding author
Phone: 82-2-2220-0491; Fax: 82-2-2291-0838;
E-mail: bskim@hanyang.ac.kr

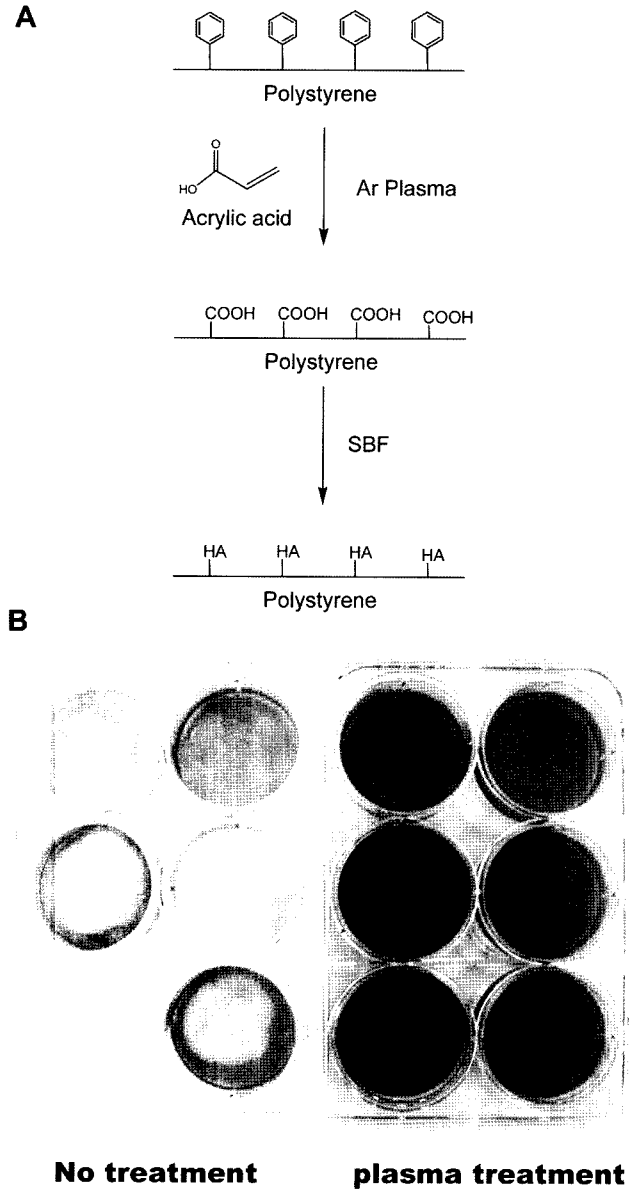

Fig. 1. A. The plating surface modification scheme. **B.** Confirmation of HA coating.
Untreated (left) and plasma treated (right).

(SBF) (Fig. 1A). 96-Well tissue culture plates were plasma treated in a cylindrical (10 cm internal diameter and 50 cm length) glass reactor capped with two earthed brass flanges [39]. The upper electrode and the reactor wall were grounded. The reactor was connected to a vacuum pump and liquid nitrogen cold trap. Radio frequency power (13.56 MHz) was coupled to the reactor *via* an impedance matching unit and an external copper coil. The plate was placed on the grounded electrode, and the system was evacuated to 0.1 torr. Argon was introduced into the chamber at a flow rate of 50 sccm, while the chamber pressure was maintained at 0.2 torr. Subsequently, plasma was generated with 50 W of electric power for 1 min. After the plasma treatment, the chamber was again evacuated to 0.1 torr, and 15–50% argon/acrylic acid was introduced into the reactor. The argon/acrylic acid was present for approximately 10 min with the plasma. Finally, the plasma was turned off and argon gas was used to purge the surface of highly reactive radicals, which could cause premature contamination of the surface. The reactor was returned to atmospheric pressure and the plate, now covered with thin plasma-polymerized films, was removed. SBF (5×) was prepared by sequentially dissolving $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$, and $K_2HPO_4 \cdot 3H_2O$ in distilled deionized water [22]. The pH was adjusted to 6 with HCl to increase the solubility. When the solution had cleared, Na_2SO_4, KCl, and NaCl were added. The final pH (6.5) was adjusted with 1 M NaOH or 1 M HCl [8]. The plasma-treated plate was immersed into the saturated 5× SBF for one week. The HA coating was confirmed by dyeing with trypan blue solution.

hBMMSC Culture

After receiving approval from the Department of Orthopaedic Surgery at Korea University, bone marrow aspirate was obtained from a hematologically normal, informed, and consenting patient undergoing a routine total hip replacement surgery. Primary hBMMSC cultures were established as previously described [12, 36]. In brief, bone marrow was mixed with an equal volume of phosphate-buffered saline (PBS, Sigma, St. Louis, MO, U.S.A.). The mixture was centrifuged on Ficoll-Paque density gradient (Amersham Biosciences, Arlington Heights, IL, U.S.A.) for 20 min at 1,500 rpm. Bone marrow mononuclear cells (BMMNCs) were isolated from the buffy coat layer between the Ficoll-

Table 1. Concentrations of factors used in each experimental group.

Experimental group	AC (mg/l)	βG (mM)	BMP-2 (ng/ml)	HA
1	50	10	100	Coated
2	0	10	100	Coated
3	50	0	100	Coated
4	50	10	0	Coated
5	50	10	100	Uncoated
6	0	0	100	Coated
7	0	10	0	Coated
8	0	10	100	Uncoated
9	50	0	0	Coated
10	50	0	100	Uncoated
11	50	10	0	Uncoated
12	0	0	0	Coated
13	0	0	100	Uncoated
14	0	10	0	Uncoated
15	50	0	0	Uncoated
16	0	0	0	Uncoated

Paque reagent and blood plasma component. BMMNCs were washed three times in PBS and plated in culture dishes (100-mm diameter, Corning Incorporated, NY, U.S.A.). hBMMSCs were obtained by culturing the BMMNCs in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersburg, MD, U.S.A.) containing 10% (v/v) fetal bovine serum (FBS, Gibco BRL), 1% (v/v) penicillin-streptomycin (Gibco BRL) in humidified air with 5% CO_2 at 37°C. Non-adherent cells were removed and adherent cells (hBMMSCs) were cultured and expanded for further experiments. The hBMMSCs from the first passage were used in the following experiments. Cells were counted with a hemacytometer. hBMMSCs (5×10^3 cells/well) were plated in 96-well plates and cultured with DMEM plus the supplements shown in Table 1. Six independent trials were carried out for each experimental condition.

Experimental Design and Statistical Analysis

A 2^4 factorial design with four variables (Table 1) was used to investigate the effects of each variable alone and in combination. An HA substrate and bone morphogenic protein-2 (BMP-2), βG, AC, and the culture medium were the variables. ALP activity, calcium deposition, and OPG and OPN expression were the responses to be measured. Design-Expert (version 7, Stat-Ease Inc.) was used to investigate the interactions among the variables [5].

To investigate the effects of the 4 factors on hBMMSC osteogenic differentiation, the ALP activity, OPG and OPN secretion, and calcium deposition were measured after 7 days of culture. Using standard methods of analyzing factorial design experiments and analysis of variance (ANOVA), the significance of each treatment combination was determined. Statistical analysis obtained from the 2-level (treatment and no treatment) factorial design experiment reported the magnitudes of the effects and their respective P values for single-factors (main) and higher-order interactions. The magnitudes of the effects were used to determine whether a treatment had a negative or positive response. This approach allowed us to score significant differences between measured outcomes (Figs. 2, 3, 4, and 5).

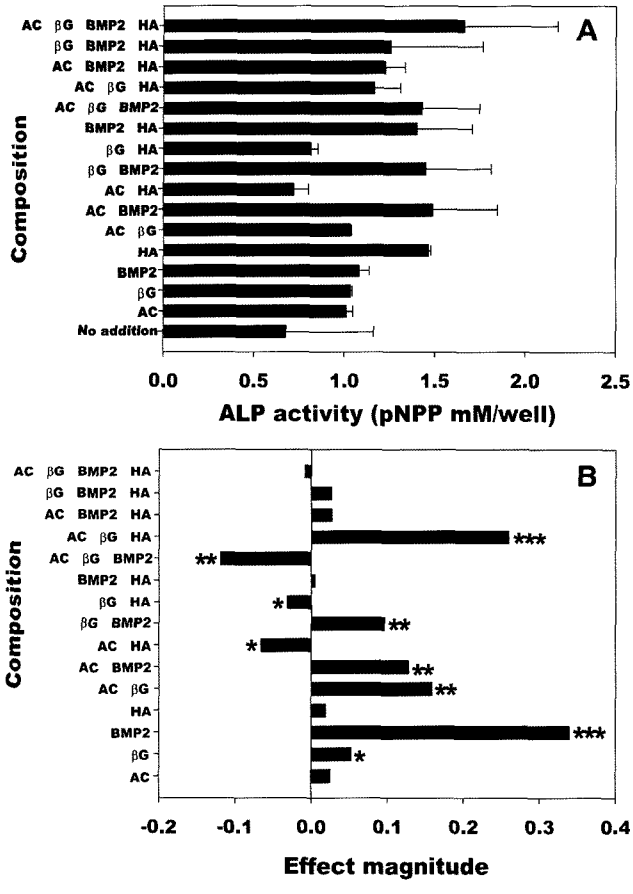

Fig. 2. ALP activity.
A. Quantification of ALP activity. The values represent the mean±standard deviation. B. The effects of the variables (AC, βG, BMP-2, and HA) and interactions between the variables on ALP activity of cultured hBMMSCs. *p<0.05; **p<0.01; ***p<0.001.

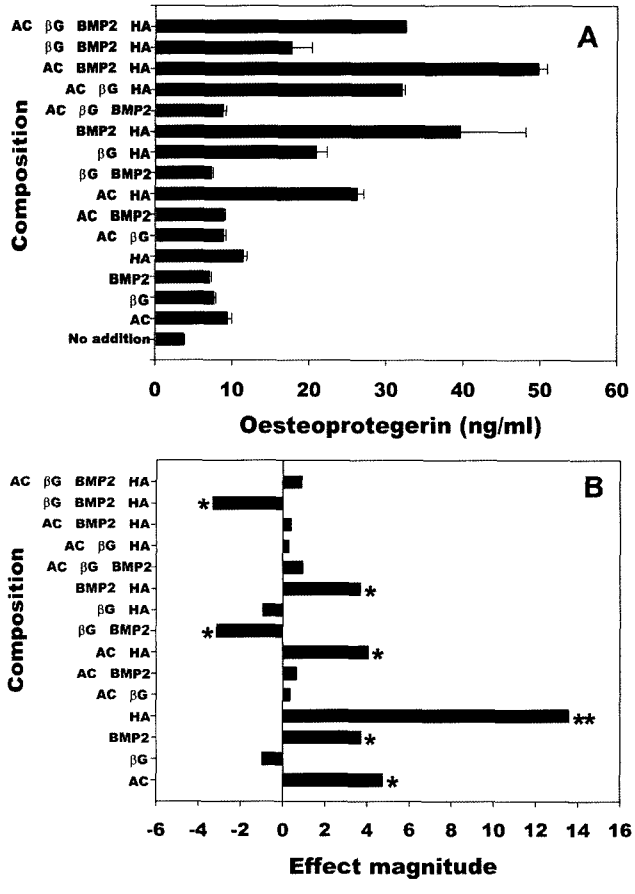

Fig. 3. Osteoprotegerin secretion.
A. Quantification of OPG secretion. The values represent the mean±standard deviation. B. The effects of the variables (AC, βG, BMP-2, and HA) and interactions between the variables on OPG secretion of cultured hBMMSCs. *p<0.05; **p<0.01.

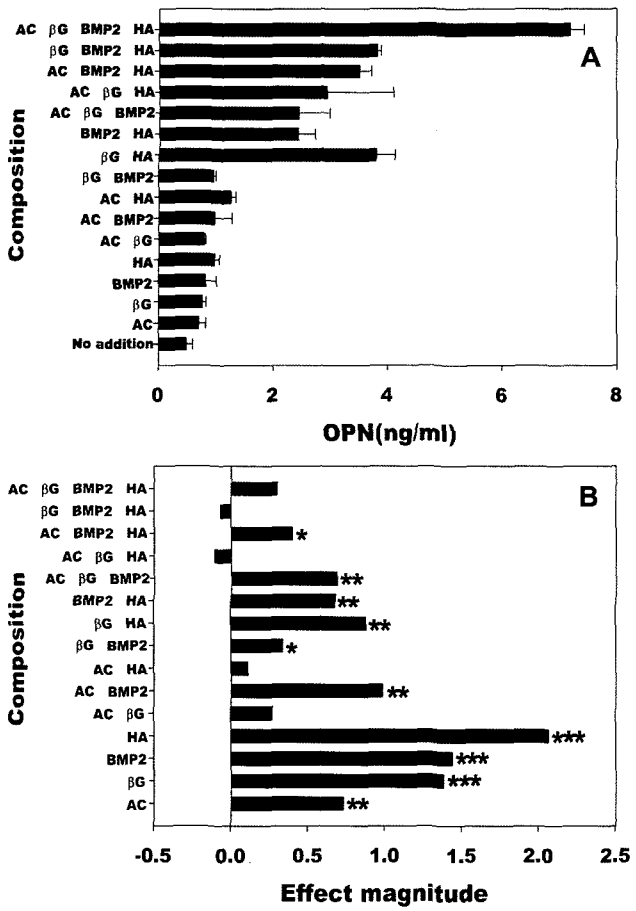

Fig. 4. Osteopontin secretion.
A. Quantification of OPN secretion. The values represent the mean±standard deviation. **B.** The effects of the variables (AC, βG, BMP-2, and HA) and interactions between the variables on OPN secretion of cultured hBMMSCs. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

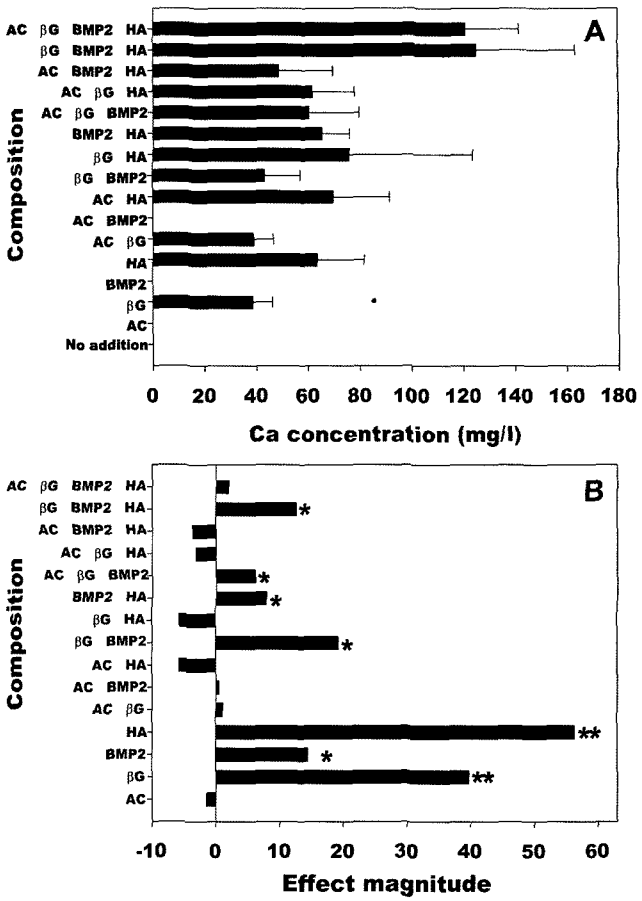

Fig. 5. Calcium deposition.
A. Quantification of calcium deposition. The values represent the mean±standard deviation. **B.** The effects of the variables (AC, βG, BMP-2, and HA) and interactions between the variables on calcium deposition of cultured hBMMSCs. *$p<0.05$; **$p<0.01$.

Quantitative Analyses

After 7 days of culture, the alkaline phosphatase (ALP) activity [1, 29] and the calcium, osteoprotegerin (OPG), and osteopontin (OPN) concentrations were determined. To determine ALP activity ($n=6$), 50 μl of lysis buffer (Cell Culture Lysis Reagent 5×, Promega) was added to each well, and the lysates were cleared by centrifugation for 10 min at 13,000 rpm using an ultracentrifuge (Micro 17 R, Hanil Science Industrial, Seoul, Korea). Each sample was incubated with 150 μl of alkaline phosphatase substrate containing *p*-nitrophenylphosphate (*p*NPP, Sigma) at 37°C for 30 min. The reaction was stopped by adding a ¼ volume of 3 N NaOH. The absorbance of the samples was read at 405 nm using an ELISA plate reader.

To quantify the calcium deposition, the culture plates were rinsed twice with PBS and homogenized with 0.6 N HCl, at which point, calcium was extracted by shaking for 4 h at 4°C [21]. The lysate was then centrifuged at 1,000 ×*g* for 5 min, and the calcium content of the supernatant was determined. The calcium concentration in the lysates was quantified spectrophotometrically with cresolphthalein complexone (Sigma). Three minutes after adding the reagents, the absorbance at 575 nm was read using a microplate reader (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland). The calcium concentration was calculated from a standard curve generated with a serial dilution of a calcium standard solution (Sigma).

The OPG and OPN concentrations in the culture supernatants were determined with ELISA kits for each. ELISA plates (NUNC, Polylabo, Strasbourg, France) were coated with capture monoclonal antibodies, and blocked with bovine serum albumin (1 w/v %) and sucrose (5 w/v %) for 1 h. Osteoprotegerin and osteopontin diluted samples were detected using anti-human osteoprotegerin and osteopontin polyclonal antibodies. Then, streptavidin-conjugated horseradish peroxidase was added to the plates. The enzyme (peroxidase) and substrate (tetramethylbenzidine) were added and incubated for 20 min. The enzyme reaction

was stopped by adding an acidic solution. The absorbance was read at 450 nm using an ELISA plate reader (PowerWave X340, Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.). The amount of osteoprotegerin and osteopontin was determined from a calibration curve with known concentrations of osteoprotegerin and osteopontin.

RESULTS AND DISCUSSION

HA coating of the culture plates was dramatically enhanced by plasma treatment, as measured by staining with a hydrophilic dye. There was more abundant staining on plasma-treated plates than untreated plates (Fig. 1B). The enhanced HA coating may be attributed to the carboxyl groups introduced to the plates, since negatively charged groups strongly induce HA formation in SBF incubation [41].

The highest ALP activity was seen with AC+βG+BMP-2+HA treatment, whereas the lowest ALP activity was seen with AC+HA treatment (Fig. 2A). BMP-2 and combined AC+βG, AC+BMP-2, βG+BMP-2, and AC+βG+HA had positive effects on ALP activity. BMP-2 alone showed the highest positive effect (Fig. 2B). The interaction between AC, βG, and HA had the second highest positive effect on ALP activity.

The highest OPG secretion was induced by AC+BMP-2+HA, whereas the lowest OPG secretion was achieved in the BMP-2 group (Fig. 3A). Although the lowest, BMP-2 still showed a significantly positive effect on OPG secretion (Fig. 3B). AC, BMP-2, HA, AC+HA, and BMP-2+HA all showed positive effects on OPG secretion (Fig. 3B). HA had the highest positive effect (Fig. 3B).

AC+βG+BMP-2+HA induced the largest amount of OPN secretion, whereas the AC group induced the smallest amount of OPN secretion (Fig. 4A). Again, AC had a significantly positive effect on OPN secretion (Fig. 4B). AC, βG, BMP-2, HA, AC+BMP-2, βG+BMP-2, βG+HA, BMP-2+HA, AC+βG+BMP-2, and AC+BMP-2+HA all had positive effects on OPN secretion (Fig. 4B). HA had the highest positive effect on OPN secretion (Fig. 4B).

Osteogenic cells that have differentiated from mesenchymal stem cells express several bone-type extracellular matrix proteins, such as OPN, OPG, bone sialoprotein, and osteocalcin, and produce high levels of ALP. High-level ALP expression is required to mineralize skeletal tissue, and is induced during early osteoblast differentiation [2, 10, 34]. Among the many proteins expressed early in osteogenesis, ALP, OPN, and OPG are important early indicators of a cell's osteogenic capacity. In this study, BMP-2 treatment had a strong positive effect on ALP, OPN, and OPG secretions. This suggests that BMP-2 treatment may affect early osteogenic differentiation of hBMMSCs *in vitro*.

Calcium deposition was highest in the βG+BMP-2+HA group, whereas AC+BMP-2, BMP-2, and AC treatment induced no calcium deposition (Fig. 5A). βG, BMP-2, HA, βG+BMP-2, BMP-2+HA, AC+βG+BMP-2, and βG+BMP-2+HA had positive effects on calcium deposition (Fig. 5B). HA gave the highest positive effect on calcium deposition (Fig. 5B). HA and βG are known to promote calcification of the extracellular matrix produced by osteoblasts [13, 32]. Consistent with previous reports [13, 32], the present study shows that HA and βG have strong positive effects on calcium deposition by hBMMSCs (Fig. 5B).

In summary, BMP-2 had the highest positive effect on the expression of ALP and OPG by hBMMSCs, which are early indicators of osteogenic differentiation. HA predominantly promoted calcium deposition, which occurs at the late stage of osteogenic differentiation [38]. The interaction between AC, βG, and HA had the second highest positive effect on ALP activity.

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