

Bone morphogenetic protein 2-enhanced osteogenic differentiation of stem cell spheres by regulation of Runx2 expression

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Abstract. Bone morphogenetic protein 2 (BMP-2) is a growth factor that is used to induce osteogenic differentiation in stem cells. The present study assessed the effects of BMP-2 on stem cell spheroid morphology, viability and osteogenic differentiation. Stem cell spheres were constructed and treated with BMP-2 at predetermined concentrations (0-100 ng/ml) using concave microwells. Cell viability was qualitatively and quantitatively analyzed via microscopy and a water-soluble tetrazolium salt assay kit, respectively. Alkaline phosphatase activity was assessed and an anthraquinone dye for calcium deposit evaluation was performed to determine osteogenic differentiation. The expressions of (runt-related transcription factor 2) and collagen 1 were also determined via quantitative PCR. Spherical shapes were formed using concave microwells on day 1, which were maintained up to day 21. On day 1, the relative cell viability of 0, 10 and 100 ng/ml BMP-2 treated cells was 100.0±1.9, 97.3±4.4 and 101.3±2.6%, respectively. Significantly higher values for alkaline phosphatase activity were determined in the 100 ng/ml treated group when compared with the control group. Additionally, Runx2 mRNA levels were significantly higher in the 100 ng/ml BMP-2 group compared with the control group, as determined via quantitative PCR. The results of the present study indicated that BMP-2 enhanced the differentiation of stem cell spheres, which was demonstrated by increased alkaline phosphatase activity and Runx2 expression.

Introduction

Bone marrow mesenchymal stem cells are known to have multi-lineage differentiation potential and are used for various therapeutic purposes (1). Bone morphogenetic protein 2 (BMP-2) has been widely applied for the regeneration of destructed bone (2). More recently, BMP-2 has been applied for osteogenic differentiation of stem cells (3). Application of BMP-2 enhanced *in vitro* mineralization (4). Moreover, it has been reported that type 2 diabetes mellitus may impair differentiation of bone marrow mesenchymal stem cells. BMP-2 has promoted osteogenesis of bone marrow mesenchymal stem cells in type 2 diabetic rats through the Wnt signaling pathway (5).

The traditional three-dimensional cultures have been of great interest because they can mimic *in vivo* conditions (6). The three-dimensional culture system has produced higher cell survival for stem cells (7) and has a better capability to maintain inherent cell characteristics (8). Three dimensional spheres made from bone marrow-derived stem cells were able to differentiate into various types of cells (9). More recently, three-dimensional human bone marrow mesenchymal stem cells have been evaluated osteogenesis (10). The three-dimensional culture technique had the advantage of enhanced differentiation of stem cells into osteoblasts when compared with a two-dimensional culture technique (11). This study was performed to evaluate the effects of BMP-2 on proliferation, osteogenic potential, and protein expression using cell spheres composed of bone marrow mesenchymal stem cells and to analyze the feasibility of the stem cell spheroid in tissue regeneration.

Materials and methods

Cell spheres using bone marrow mesenchymal stem cells. Ethical approval was granted regarding the present study through the Institutional Review Board of Seoul St Mary's Hospital, College of Medicine, the Catholic University of Korea (number: KC18SESI0083), and all experiments were carried out following the relevant guidelines. Human bone marrow mesenchymal stem cells (BMSCs, Catholic MASTER cells) were obtained from the Catholic Institute of Cell Therapy (CIC, Seoul, Korea). Isolation and propagation of the BMSCs were performed following previously

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reported methods (12). CIC verified that all samples showed >90% positive CD 73 and CD 90 expression. We seeded the cells on a culture dish. We removed the cells that were not attached to the dish. We refreshed the culture medium every 2 or 3 days, and grew the cells in the incubator with 95% O₂ and 5% CO₂ at 37°C.

Fig. 1 shows an overview of the study's design. We used commercially available concave microwells (H389600, StemFIT 3D; MicroFIT) to fabricate stem cell spheres. We loaded a total of 1×10^6 cells in each well and evaluated the cell response. We treated cell spheres made of bone marrow mesenchymal stem cells with BMP-2 at predetermined concentrations of 0, 10 and 100 ng/ml. We evaluated the morphological characteristics using an inverted microscope (Leica DM IRM, Leica Microsystems). Morphological evaluation of the spheres was conducted on days 1, 3, 7, 14, and 21.

Determination of cellular viability. We cultured stem cell spheres in osteogenic media. We used the commercially available two-color assay based on plasma membrane integrity and esterase activity (Live/Dead Kit assay, Molecular Probes) for qualitative analysis of the stem cell spheres on days 1, 3, 5, and 7. We also performed a quantitative cellular viability test using an assay kit based on water-soluble tetrazolium salt (Cell Counting Kit-8). Experiments were carried out in triplicate.

Level of alkaline phosphatase activity and calcium deposition. The level of alkaline phosphatase activity and an anthraquinone dye assay for calcium deposit evaluation were used to assess osteogenic differentiation on day 14 (13). Alkaline phosphatase activity is reported to an early marker of osteogenic differentiation (14). Highest alkaline phosphatase activity in preosteoblast was noted on day 14 (15). We obtained cell spheres grown on culture plates with osteogenic media. We used a commercially available kit (K412-500, BioVision, Inc.) for the evaluation of level of alkaline phosphatase activity.

We used an anthraquinone dye assay for calcium deposit evaluation to assess osteogenic differentiation on days 7, 14, and 21. We stained the stem cell spheres with Alizarin Red S for 30 min at room temperature after washing and fixation procedures. The quantification of the bound dyes was performed on day 21. The assays were performed three times.

Evaluation of Runx2 and Coll by quantitative polymerase chain reaction. We harvested the cells on day 7. We isolated total RNA using purification (Thermo Fisher Scientific, Inc.); RNA was used as a template for reverse transcription using SuperiorScript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.), and we determined quantities were within ratios of absorbance at 260 and 280 nm spectrophotometrically (ND-2000, Thermo Fisher Scientific, Inc.) on day 7.

We detected mRNA expression by quantitative polymerase chain reaction. We designed the sense and antisense primers based on GenBank. The primer sequences were in the followings: Runx2 forward 5': AATGATGGTGTGACGCTGA-3'; reverse 5': TTGATACGTGTGGGATGTGG-3'; Coll forward 5': CCAGAAGAACTGGTACATCAGCAA-3', reverse 5': CGC

CATACTCGAACTGGAATC-3', β -actin forward 5': TGG CACCCAGCACAATGAA-3', and reverse 5': CTAAGTCAT AGTCCGCTAGAAGCA-3'. Normalization was performed by applying β -actin as a housekeeping gene. The experiments were performed in three times.

Statistical analysis. We presented the results as means \pm standard deviations of the data. Shapiro-Wilk test were performed to conduct the tests of normality and we checked the Levene's tests to evaluate the equal of variances. We performed two-way analysis of variance to evaluate the effects of concentration and time points. We tested the differences among groups by applying one-way analysis of variance with Tukey's post hoc test (SPSS 12 for Windows, SPSS Inc.). The significance level was set at $P < 0.05$.

Results

Formation of cell spheres with human bone marrow-derived stem cells. We noticed a spherical shape of stem cell aggregates on day 1 (Fig. 2). No significant morphological change of the cell spheres cultured in osteogenic media was observed with the addition of BMP-2. Shape on days 3, 7, 14, and 21 is shown in Fig. 2, and no noticeable changes were noted with longer incubation time.

Cellular viability. The qualitative results on the viability of cell spheres were analyzed using a Live/Dead Kit assay at day 1, as shown in Fig. 3A. In all cases, we noticed that most of the cells in the spheres presented green fluorescence, indicating live cells. We did not see any noticeable changes with longer incubation time (Fig. 3B-D).

The quantitative value for cellular viability on days 1, 3, 5, and 7 is shown in Fig. 4. The relative cell viability assay values were 100.0 ± 1.9 , 97.3 ± 4.4 , and $101.3 \pm 2.6\%$ for BMP-2 at 0, 10, and 100 ng/ml on day 1, respectively ($P > 0.05$).

Level of alkaline phosphatase activity and calcium deposition. The results of the alkaline phosphatase activity assays on day 14 are shown in Fig. 5. The absorbance values at 405 nm at day 14 for BMP-2 at 0, 10, and 100 ng/ml were 0.081 ± 0.001 , 0.084 ± 0.007 , and 0.103 ± 0.006 , respectively. There were significantly higher values for BMP-2 groups at 100 ng/ml concentration when compared with the control group ($P < 0.05$).

The results of the mineralization assay at days 7, 14, and 21 are shown in Fig. 6A. Mineralized extracellular deposits were evenly noted in each group. The quantification results showed that there were significantly higher values for BMP-2 groups at 100 ng/ml concentration when compared with the control (Fig. 6B; $P < 0.05$).

Evaluation of Runx2 and Coll by quantitative polymerase chain reaction. We saw significantly higher expression of mRNA levels of Runx2 using quantitative polymerase chain reactions in BMP-2 groups at 100 ng/ml concentration when compared with the control ($P < 0.05$, Fig. 7A). The results showed that application of BMP-2 at a concentration of 100 ng/ml did not produce significant changes in Coll expression when compared with the control ($P > 0.05$, Fig. 7B).

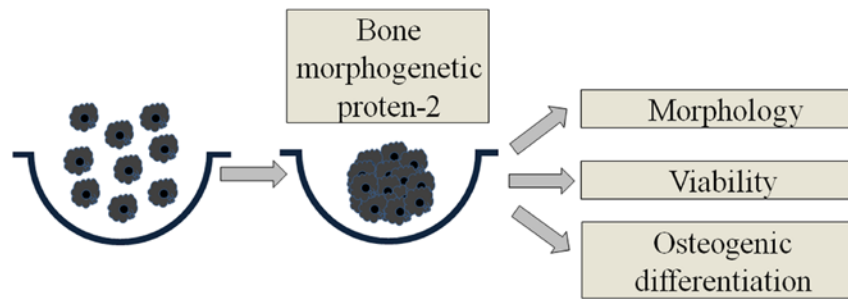


Figure 1. Overview of study design.

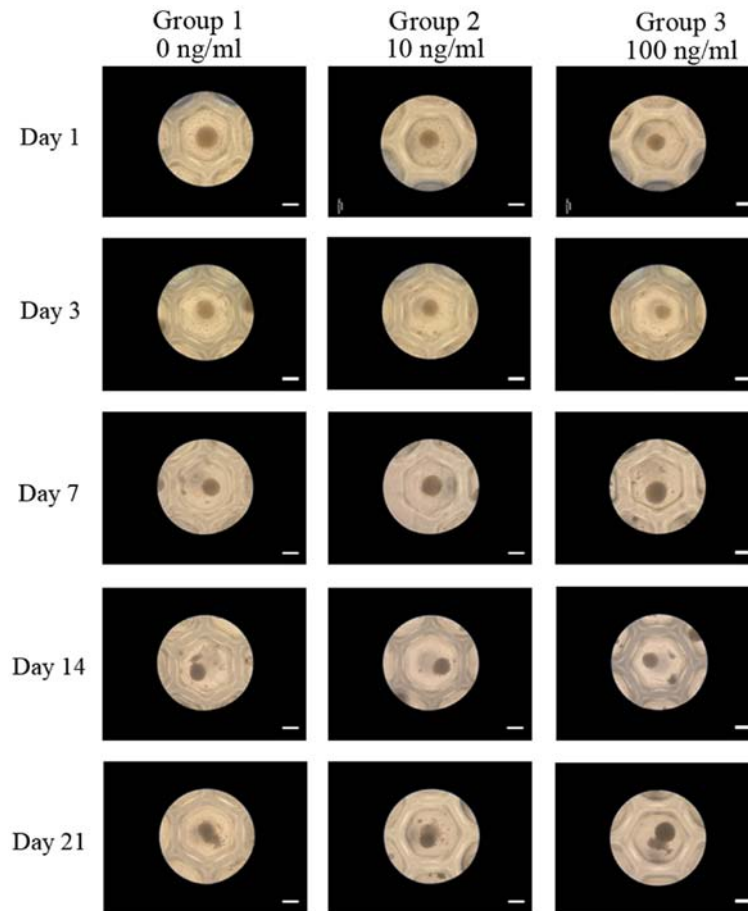


Figure 2. Stem-cell sphere morphology at days 1, 3, 7, 14 and 21 (scale bar, 200 μ m).

Discussion

This report discusses the effects of BMP-2 on cellular viability and osteogenic differentiation using cell spheres of stem cells. Our study showed that the application of BMP-2 increased alkaline phosphatase activity and increased Runx2 at 100 ng/ml.

Bone marrow mesenchymal stem cells may enhance bone regeneration through secretion of growth factors or through direct differentiation into bone cells (16). The results of this study suggest combination therapy using stem cells and BMP-2 with a three-dimensional approach. The combined approach of osteoconductive scaffolds and osteoinductive growth factors may have synergistic effects on osteogenesis (17). In a

previous report, the application of a combined approach with bone marrow mesenchymal stem cells, BMP-2 and hydroxyapatite led to successful treatment of critical-sized defects in human beings (18). Bone marrow stem cells were loaded into a hydrogel system by enzyme-catalyzed crosslinking, and the addition of bone BMP-2 led to the improvement of osteogenic potential (19). Moreover, transient transfection of stem cells with BMP-2 expressing plasmids was applied. The transfected cells were loaded into a three-dimensional hydrogel system, and higher expression of osteogenic transcription factor was achieved (20).

The effects of concentration of BMP-2 were evaluated in previous studies (21-25). It was reported that BMP-2 in concentrations of 0.1, 0.5, 1, 10, 50, 100, and 300 ng/ml was

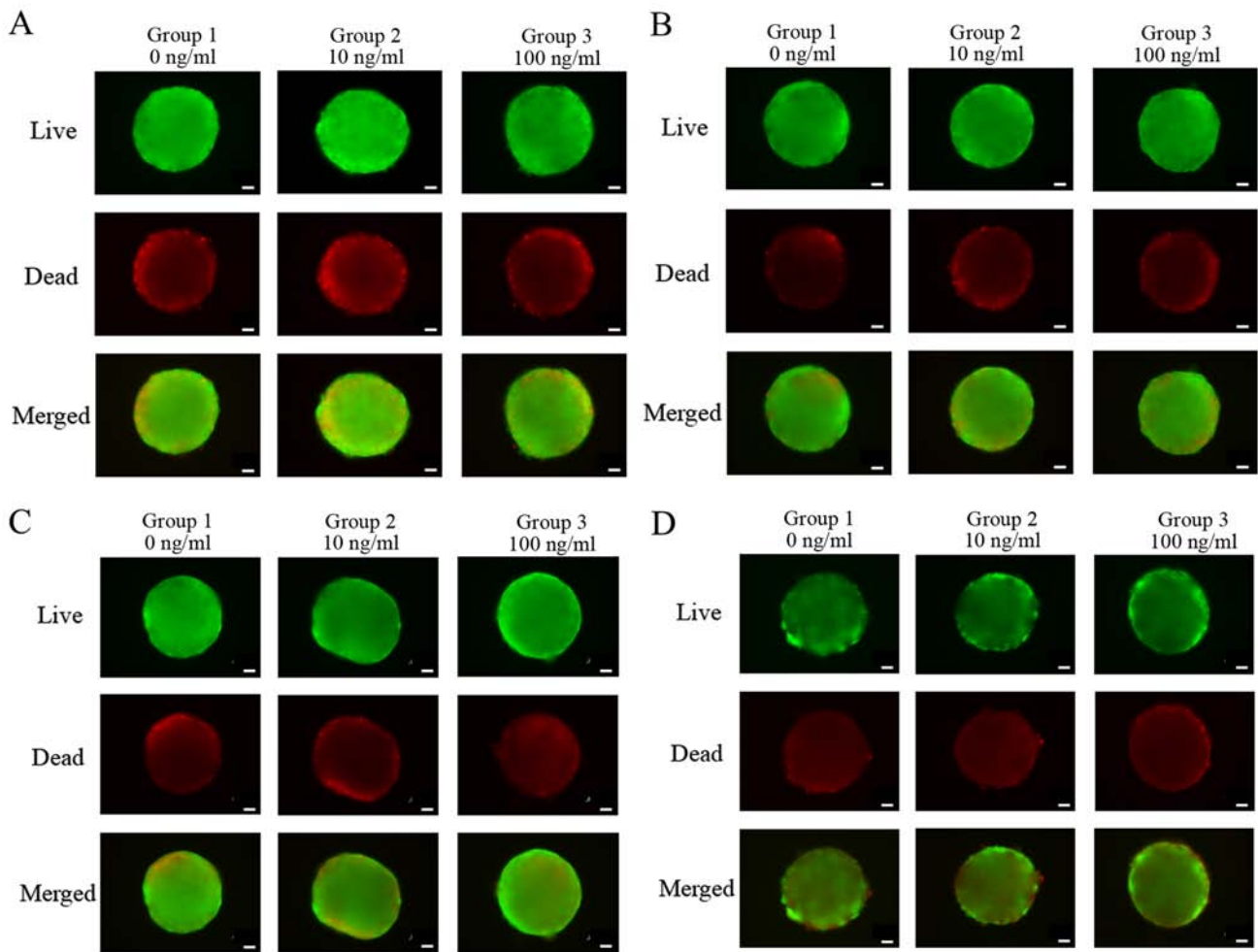


Figure 3. Live, dead, and merged images of stem cell spheres. Results are presented for days (A) 1, (B) 3, (C) 5 and (D) 7 (scale bar, 50 μ m).

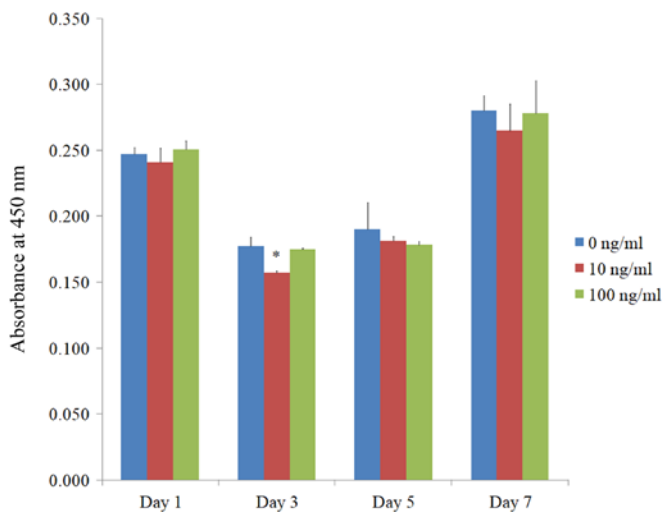


Figure 4. Cellular viability determined using a Cell Counting Kit-8 assay on days 1, 3, 5 and 7. * $P < 0.05$ vs. control at day 3.

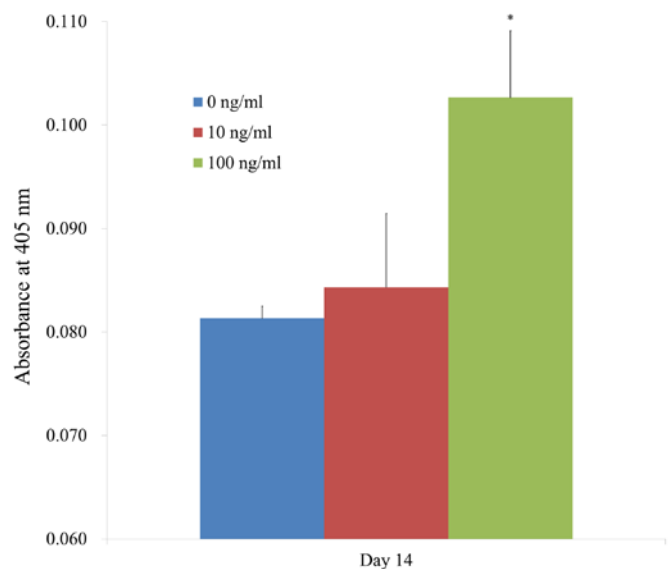


Figure 5. Alkaline phosphatase activity on day 14. * $P < 0.05$ vs. control.

evaluated for osteoblast differentiation, and a dose of 50 ng/ml or above of BMP-2 was effective (21). Various concentrations of BMP-2, including 20, 50, and 100 ng/ml, were applied, and it was suggested that 50 ng/ml of BMP-2 may be optimal (22). The addition of 60 ng/ml BMP-2 exhibited

enhanced osteoblast differentiation by regulating the expression of phospho-Smad1/5/8 (23). In rat animal models, BMP-2 in a concentration of 50 ng/ml increased the radiographic density of bone defects (25). However, preconditioning of

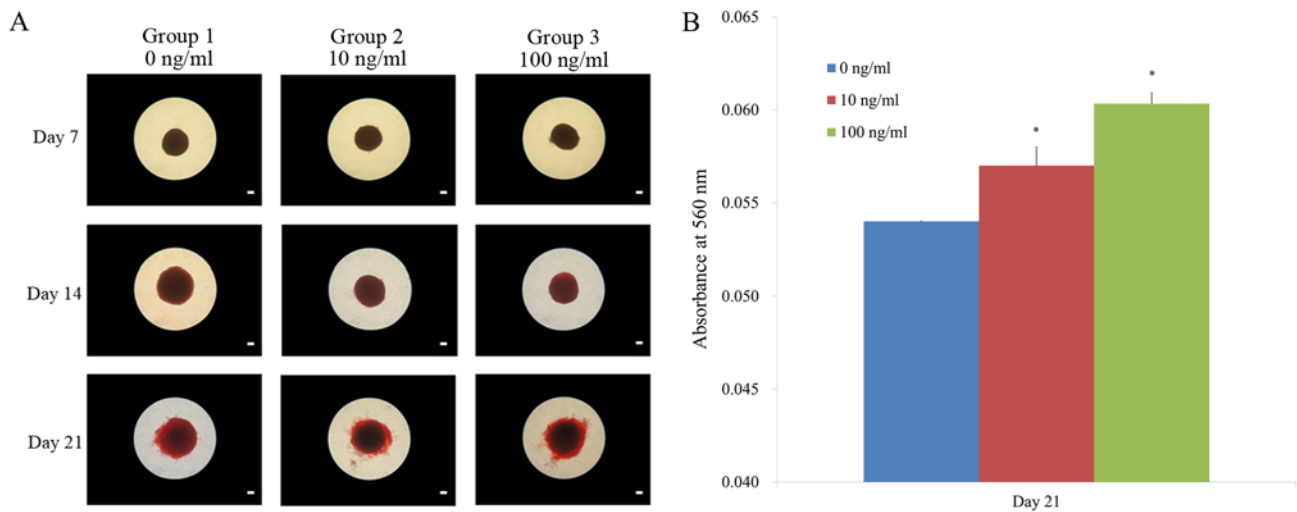


Figure 6. Results of Alizarin Red S staining. Scale bars in images of (A) represent 200 μ m. (B) Results were quantified on day 21. * $P < 0.05$ vs. control.

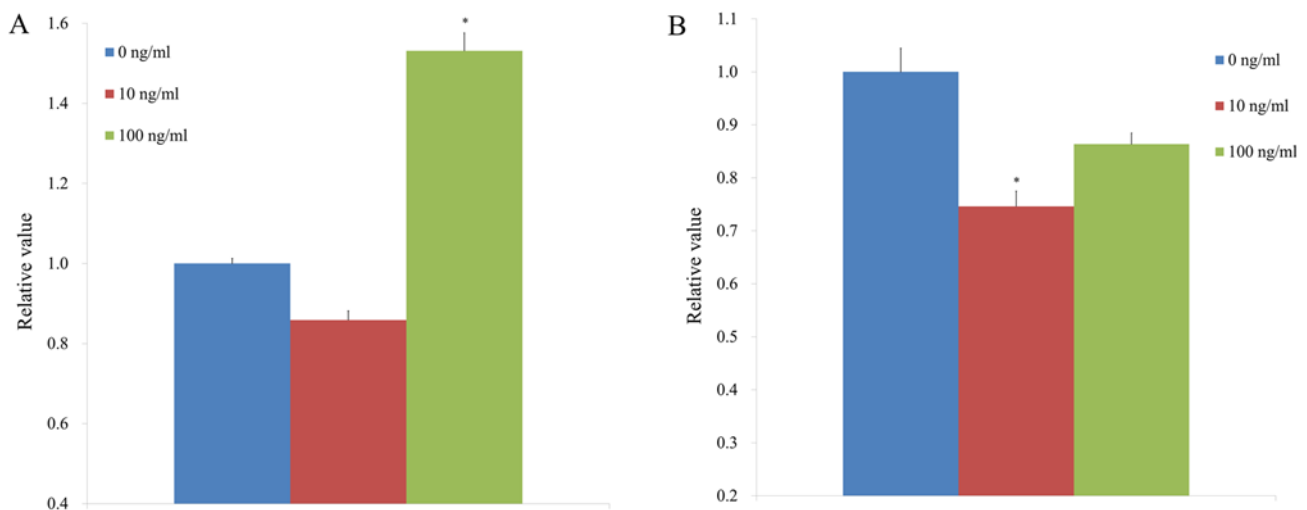


Figure 7. Results of total RNA extraction and quantification by reverse transcription-quantitative PCR. Expressions of (A) Runx2 and (B) Col1 are presented. * $P < 0.05$ vs. control.

mesenchymal stem cells with lower concentrations of 10 and 20 ng/ml of BMP-2 enhanced osteogenic differentiation (24). In a previous report, the application of BMP-2 significantly increased gene expression of Runx2 and Col1 (24). In this study, the highest expression of Runx2 was noted at 100 ng/ml; however, no significant change of Col1 expression was noted at 100 ng/ml. Moreover, a previous report also showed that BMP-2 induced osterix expression, but it was independent of Runx2 expression (26). In a previous report, the application of BMP-2 resulted in increased bone sialoprotein production (27). The overall effects of BMP-2 or maximal effective dose of BMP-2 may show differences because of differences in types of cells, culturing condition, and culturing period (28).

In this study, stem cell spheres were made without using a scaffold and their morphology was maintained up to the experimental time points. Scaffolds made of animal-derived or chemical biomaterials are widely utilized for stem cell-based tissue regeneration (29). More recently,

advances in three-dimensional printing technology have made it possible to utilize scaffold-free spheroid-based bioprinting (30). It seems that an exogenous scaffold-free approach may have the advantage of long-term safety (29). A previous report used a scaffold-free structure with a mixture of cell types using three-dimensional printing technology, and the larger amount of stem cells resulted in greater strength (31).

The effects of stem cell spheres treated with BMP-2 may be further enhanced. In a previous study, researchers sequentially applied BMP-2 and basic fibroblast growth factor to achieve synergistic promotion of osteogenesis (32). Similarly, retinoic acid has been shown to increase the effect of BMP-2 on osteogenic differentiation of stem cells (33). BMP-2 and Forkhead c2 synergistically enhance osteogenic differentiation and mineralization of stem cells (34). Moreover, mesenchymal stem cells were treated with endothelial cells encapsulated in collagen/fibrin hydrogels to increase stem cell functionality (35).

Collectively, this study shows that the application of BMP-2 increases alkaline phosphatase activity and Runx2 expression in stem cell spheres at 100 ng/ml. This research suggested that the use of BMP-2 enhanced the differentiation of stem cell spheres, which was demonstrated by increased alkaline phosphatase activity and Runx2 expression. Future studies may be warranted for the use of BMP-2 with stem cell spheroids for various models, including *in vivo* bony defect models in the calvaria and mandibles. The osteogenic effects of stem cell spheroids with the different dosages of BMP-2 can be evaluated histomorphometrically by analyzing the amount of bone formation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SKM, MK and JBP conceived and designed the experiments of the present study, analyzed the data, and wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Review Board of Seoul St Mary's Hospital, College of Medicine, Catholic University of Korea (approval no. KC18SESI0083).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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