

Evaluation of the shape, viability, stemness and osteogenic differentiation of cell spheroids formed from human gingiva-derived stem cells and osteoprecursor cells

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Abstract. The present study was performed to create stem cell spheroids from human gingiva-derived stem cells and osteoprecursor cells and to evaluate the maintenance of the stemness, the viability and osteogenic differentiation of the cell spheroids. Gingiva-derived stem cells were isolated, and a total of 6×10^5 stem cells and osteoprecursor cells were seeded into concave micromolds at various ratios. Gingiva-derived stem cells and/or osteoprecursor cells formed spheroids in concave microwells. The spheroids demonstrated a smaller diameter when the number of osteoprecursor cells seeded was lower. The majority of cells in the spheroids were identified to be live cells and the cell spheroids preserved viability throughout the experimental period. The cell spheroids, which contained stem cells, were positive for stem-cell markers. Cell spheroids in concave microwells demonstrated a statistically significant increase in alkaline phosphatase activity as time progressed ($P < 0.05$). A statistically significant difference in phosphatase activity was observed in the stem cell alone group when compared with the osteoprecursor cell group at day 5 ($P < 0.05$). Mineralized extracellular deposits were observed in each group after Alizarin Red S staining. Within the limits of the present study, cell spheroids from gingival cells and osteoprecursor cells maintained shape, viability, stemness and osteogenic differentiation potential.

Introduction

There is increasing interest in the potential of stem cells as they are promising candidates for the regeneration of tissue and treatment of diseases, including diabetes mellitus, arthritis

and Parkinson's disease (1,2). Stem cells may be obtained from various tissues, including bone marrow and adipose tissue (3,4). Stem cells derived from gingiva may be useful for research and treatment of diseases as harvesting stem cells from the mandible or maxilla may be performed easily under local anesthesia (5,6). Human mesenchymal stem cells have previously been isolated and characterized from the gingiva with minimal complications (7).

Three-dimensional culture systems have demonstrated the importance of intercellular interactions in regulating stem cell self-renewal and differentiation (8,9). Physiologically relevant, three-dimensional *in vitro* models have served as biological and analytical platforms for testing novel treatments and drug delivery systems (10,11). Cell-microsphere constructs formed from human adipose-derived stem cells and gelatin microspheres were recently reported to promote stemness, differentiation and controlled pro-angiogenic potential, and this three-dimensional construct demonstrated enhanced therapeutic potential (12).

Natural bone healing following fractures is initiated by osteoblasts and mesenchymal stem cells, thus a cell combination may possess potential in tissue-engineering techniques for bone defects (13). Previous studies have used co-cultures in tissue-engineering applications as these systems more effectively model the natural tissues, both physically and biologically (14,15). Previous research has demonstrated that improved viability and function were obtained by co-culturing islet cells with stem cells in concave microwells (16). However, co-cultures of osteoblasts with other cell types have not been well established (14,17). The present study was performed to generate stem cell spheroids from human gingiva-derived stem cells and osteoprecursor cells using concave microwells and to evaluate the maintenance of stemness and viability. To the best of our knowledge, the present report is the first to evaluate the maintenance of the stemness and viability of multi-cell spheroids generated from gingiva-derived stem cells and osteoprecursor cells.

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Materials and methods

Isolation and culture of gingiva-derived stem cells. Gingiva-derived stem cells were obtained using a previously

reported method (7). Gingival tissues were harvested from 28 healthy patients during periodontal treatment from April 2012 to August 2015 at the Department of Periodontics, Seoul St Mary's Hospital, College of Medicine, The Catholic University of Korea (Seoul, Republic of Korea). The design of the present study was reviewed and approved by the Institutional Review Board of Seoul St. Mary's Hospital, College of Medicine, Catholic University of Korea, (Seoul, Korea; KC11SISI0348), and written informed consent was obtained from all patients.

Briefly, subsequent to the gingiva samples being obtained, gingival tissues were de-epithelialized, minced into 1-2-mm² fragments and digested in an α -modified minimal essential medium (α -MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing dispase (1 mg/ml) and collagenase IV (2 mg/ml; both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were incubated at 37°C in a humidified incubator with 5% CO₂ and 95% O₂ for one day. Subsequently, non-adherent cells were washed with phosphate-buffered saline (PBS; WELGENE, Inc., Daegu, South Korea) two to three times and replaced with fresh medium. Media were changed every 2-3 days.

Formation of cell spheroids from human gingiva-derived stem cells and osteoprecursor cells. Stem cell spheroids were formed in the silicon elastomer-based concave microwells (StemFIT 3D; MicroFIT, Seongnam, Korea) 600 μ m in diameter. A total of 6x10⁵ gingiva-derived stem cells and murine osteoprecursor cells (MC3T3-E1 cells; American Type Culture Collection, Manassas, VA, USA) at different ratios were seeded into the micromolds and subsequently cultured at 37°C in α -minimum essential medium (α -MEM) containing 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 μ g/ml streptomycin, 200 mM L-glutamine and 10 mM ascorbic acid 2-phosphate (all Sigma-Aldrich; Merck KGaA) to investigate cellular behavior at days 1, 3, 5, and 7. The ratios between gingiva-derived stem cells and osteoprecursor cells were as follows: 0:6 (group 1); 2:4 (group 2); 3:3 (group 3); 4:2 (group 4); and 6:0 (group 5; Fig. 1). Cell aggregation and cell spheroid formation were observed and images were captured using an inverted microscope (Leica DM IRM; Leica Microsystems GmbH, Wetzlar, Germany).

Determination of cell viability. Viability of cell spheroids was qualitatively analyzed using a Live/Dead kit (Molecular Probes; Thermo Fisher Scientific, Inc.) at days 1, 3, 5 and 7 after co-culture initiation. Cell spheroids were washed twice with PBS and suspended in 1 ml α -MEM supplemented with 2 μ l calcein acetoxymethyl ester working solution (50 mM; Molecular Probes, Eugene, OR, USA) and 4 μ l ethidium homodimer-1 (2 mM; Molecular Probes) for 15 min at room temperature. The spheroids stained with calcein acetoxymethyl ester and ethidium homodimer-1 were observed under a fluorescence microscope (Axiovert 200; Carl Zeiss AG, Oberkochen, Germany). With this assay, viable cells produce an intense, uniform, green fluorescence and dead cells demonstrate a red fluorescence.

Quantitative cell viability analysis was performed using a Cell Counting kit-8 (Dojindo Molecular Technologies, Inc.,

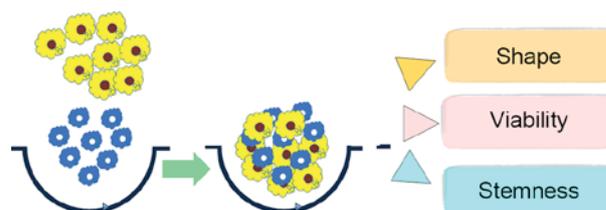


Figure 1. Schematic illustration of the procedure for generation of cell spheroids with gingival-derived stem cells and osteoprecursor cells.

Kumamoto, Japan) at days 1, 3, 5, and 7. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8; Dojindo Molecular Technologies, Inc.) was added and the spheroids were incubated for 1 h at 37°C. The spectrophotometric absorbance of the samples was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). This assay relies on the ability of mitochondrial dehydrogenases to oxidize WST-8 into a formazan product.

Evaluation of maintenance of stemness. The spheroids were retrieved at day 7 after co-culture initiation. Subsequently, the spheroids were incubated for 30 min at 37°C with human SSEA-4 (Clone MC-813-70) conjugated to NHL493 (green) and human TRA-1-60(R) (Clone TRA-1-60) conjugated to NL557 (red) antibodies (all 1:50 dilution; SC023; Live Cell Imaging Kit; R & D Systems, Inc., Minneapolis, MN, USA). The spheroids were visualized under a fluorescence microscope (Axiovert 200; Carl Zeiss AG). These antibodies were used as positive markers of human stem cells.

Alkaline phosphatase activity assays. Cell spheroids grown in osteogenic induction media (STEMPRO Osteogenesis Differentiation kit; Gibco; Thermo Fisher Scientific, Inc.) at 37°C were obtained on days 1 and 5. Alkaline phosphatase activity assays were conducted using a commercially available kit (K412-500; BioVision, Inc., Milpitas, CA, USA) at days 1 and 5, according to the manufacturer's instructions. The cells were resuspended in assay buffer, sonicated and subsequently centrifuged at 13,000 g for 3 min at 4°C to remove insoluble material. Supernatant was mixed with p-nitrophenylphosphate substrate and incubated at 25°C for 60 min. The optical density of the resultant p-nitrophenol at 405 nm was determined spectrophotometrically.

Alizarin Red S staining. At day 7, cell spheroids grown in osteogenic induction media were washed twice with PBS, fixed with 70% ethanol and rinsed twice with deionized water. Cell spheroids were stained with Alizarin Red S for 30 min at room temperature. Cell spheroids were observed and images were captured under an inverted microscope (Leica DM IRM; Leica Microsystems GmbH).

Statistical analysis. Data were presented as the mean \pm standard deviation of the experiments performed in triplicate. Student's t-tests or a two-way analysis of variance and Tukey's post hoc tests were performed to determine the differences between the groups. Statistical analyses were conducted using SPSS software for Windows (version 12; SPSS, Inc., Chicago, IL, USA).

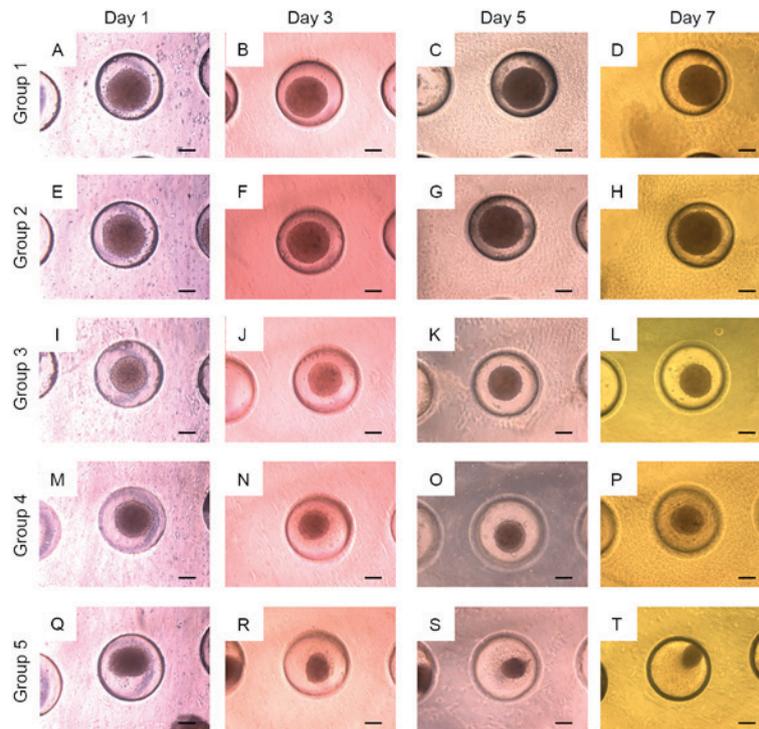


Figure 2. Morphology of stem cell spheroids at days 1, 3, 5 and 7. (A-D) Morphology of group 1 at days 1, 3, 5 and 7, respectively. (E-H) Morphology of group 2 at days 1, 3, 5 and 7, respectively. (I-L) Morphology of group 3 at days 1, 3, 5 and 7, respectively. (M-P) Morphology of group 4 at days 1, 3, 5 and 7, respectively. (Q-T) Morphology of group 5 at days 1, 3, 5 and 7, respectively. Magnification, x100; scale bar, 200 μm .

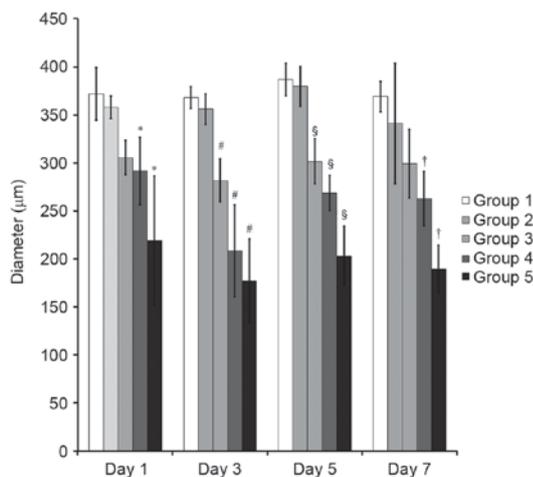


Figure 3. Diameters of the spheroids at days 1, 3, 5 and 7. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. group 1 at day 1; # $P < 0.05$ vs. group 1 at day 3; § $P < 0.05$ vs. group 1 at day 5; † $P < 0.05$ vs. group 1 at day 7.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of cell morphology. Gingiva-derived stem cells and/or osteoprecursor cells formed spheroids in concave microwells (Fig. 2). The morphology of the spheroids at days 3, 5 and 7 was similar to that at day 1. Results demonstrated that the diameter of the spheroids was smaller as the number

of osteoprecursor cells present decreased (Fig. 3). The mean spheroid diameters at day 1 were 371.9 ± 27.4 , 358.0 ± 11.8 , 305.5 ± 18.1 , 291.6 ± 35.3 and 219.2 ± 67.0 μm for groups 1, 2, 3, 4 and 5, respectively. The mean spheroid diameters at day 3 were 367.9 ± 11.3 , 356.0 ± 15.8 , 281.7 ± 22.3 , 208.3 ± 47.8 and 177.5 ± 43.6 μm for groups 1, 2, 3, 4 and 5, respectively. The mean spheroid diameters at day 5 were 386.8 ± 16.8 , 379.8 ± 20.6 , 301.5 ± 23.4 , 268.8 ± 18.4 and 203.3 ± 30.8 μm for groups 1, 2, 3, 4 and 5, respectively. The mean spheroid diameters at day 7 were 368.9 ± 15.9 , 341.2 ± 62.6 , 299.2 ± 35.9 , 262.8 ± 28.3 and 189.4 ± 25.1 μm for groups 1, 2, 3, 4 and 5, respectively. No significant change in diameter was noted within the experimental time within each group. However, a significant decrease in diameter was indicated in groups 3, 4 and 5 compared with group 1 on each respective day ($P < 0.05$), with the exception of group 3 on days 1 and 7.

Determination of cell viability. The viability results of the cell spheroids analyzed by Live/Dead kit assay are presented in Figs. 4-7. The majority of cells in the cell spheroids emitted green fluorescence throughout the experimental period, and the morphology was well maintained up to the end of the experiment. At day 7, the majority of cells in the spheroids emitted green fluorescence; however, some red fluorescence was also noted (Fig. 7).

The cell viability results obtained using a Cell Counting kit-8 after culturing at days 1, 3, 5 and 7 are presented in Fig. 8. The relative Cell Counting kit-8 assay values of groups 2, 3, 4, and 5 at day 1 were 131.3 ± 9.2 , 129.3 ± 4.2 , 115.5 ± 5.7 , and $116.4 \pm 3.5\%$, respectively, when group 1 at day 1 was considered 100% ($100.0 \pm 7.2\%$). Significant differences were seen in

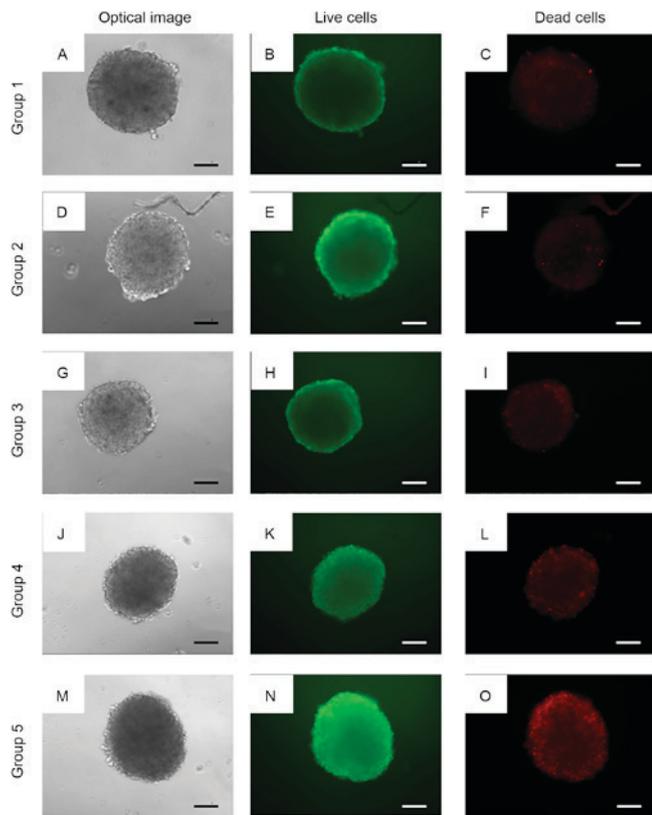


Figure 4. Live/dead cell analysis of cell spheroids at day 1 using calcein acetoxy-methyl ester and ethidium homodimer-1. Live/dead cell images of groups (A-C) 1, (D-F) 2, (G-I) 3, (J-L) 4 and (M-O) 5. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

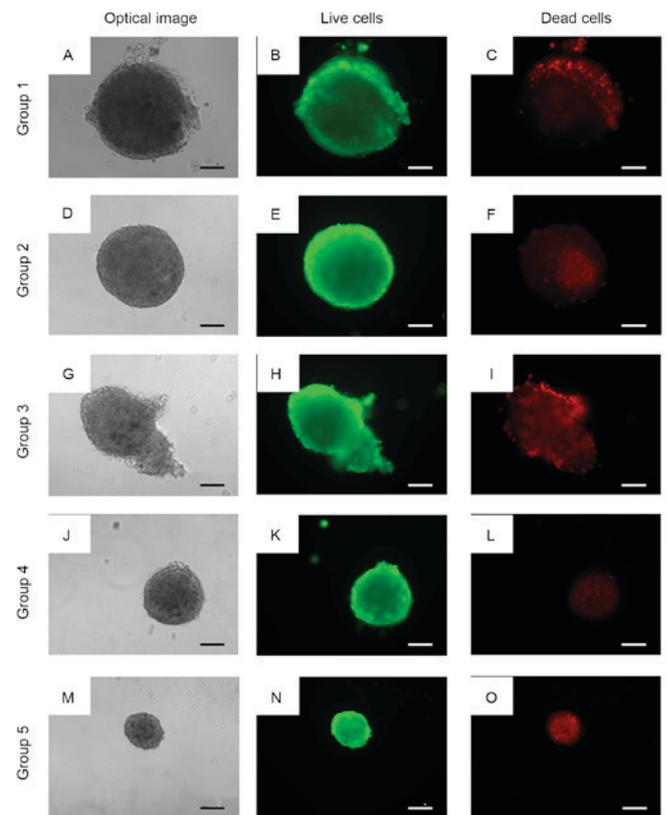


Figure 6. Live/dead cell analysis of cell spheroids at day 5 using calcein acetoxy-methyl ester and ethidium homodimer-1. Live/dead cell images of groups (A-C) 1, (D-F) 2, (G-I) 3, (J-L) 4 and (M-O) 5. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

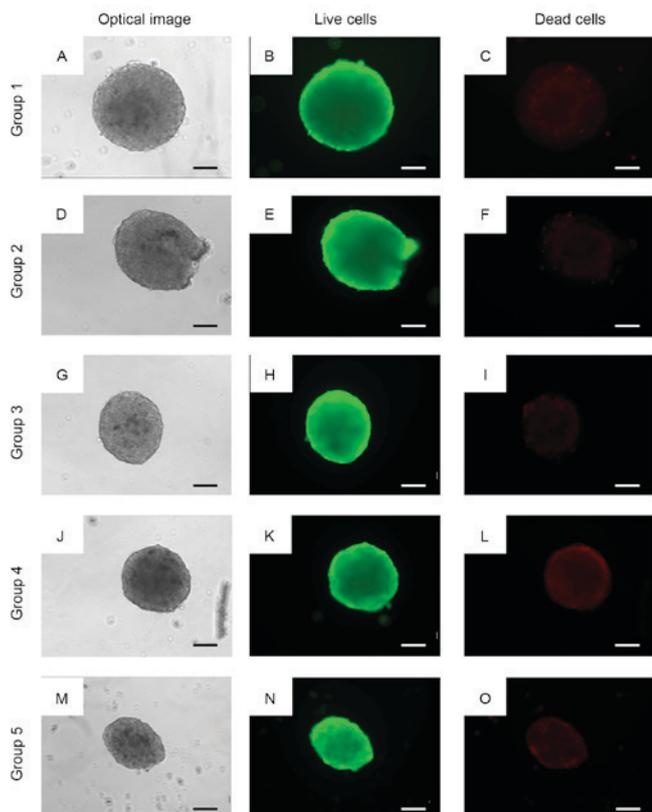


Figure 5. Live/dead cell analysis of cell spheroids at day 3 using calcein acetoxy-methyl ester and ethidium homodimer-1. Live/dead cell images of groups (A-C) 1, (D-F) 2, (G-I) 3, (J-L) 4 and (M-O) 5. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

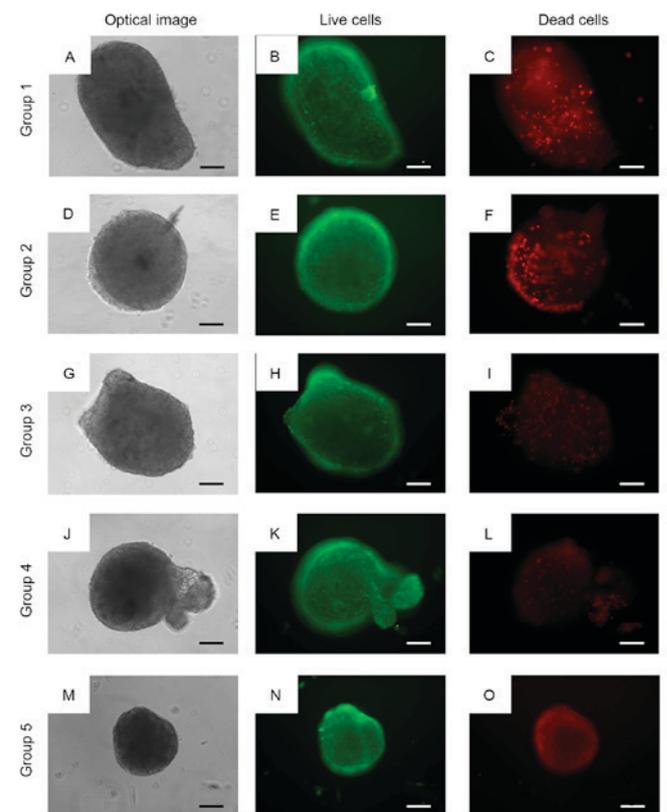


Figure 7. Live/dead cell analysis of cell spheroids at day 7 using calcein acetoxy-methyl ester and ethidium homodimer-1. Live/dead cell images of groups (A-C) 1, (D-F) 2, (G-I) 3, (J-L) 4 and (M-O) 5. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

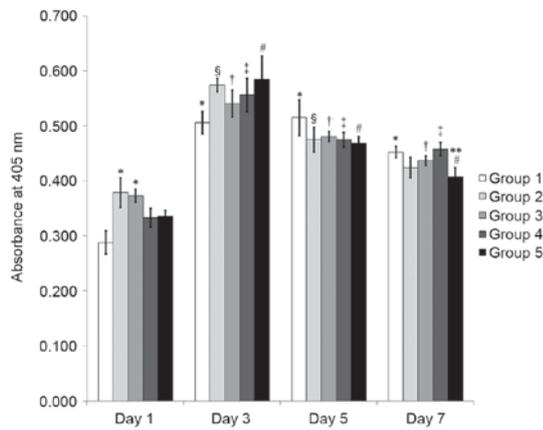


Figure 8. Viability of spheroids on days 1, 3, 5 and 7. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. group 1 at day 1; [†] $P < 0.05$ vs. group 1 at day 7; [‡] $P < 0.05$ vs. group 2 at day 1; [§] $P < 0.05$ vs. group 3 at day 1; [#] $P < 0.05$ vs. group 4 at day 1; [¶] $P < 0.05$ vs. group 5 at day 1.

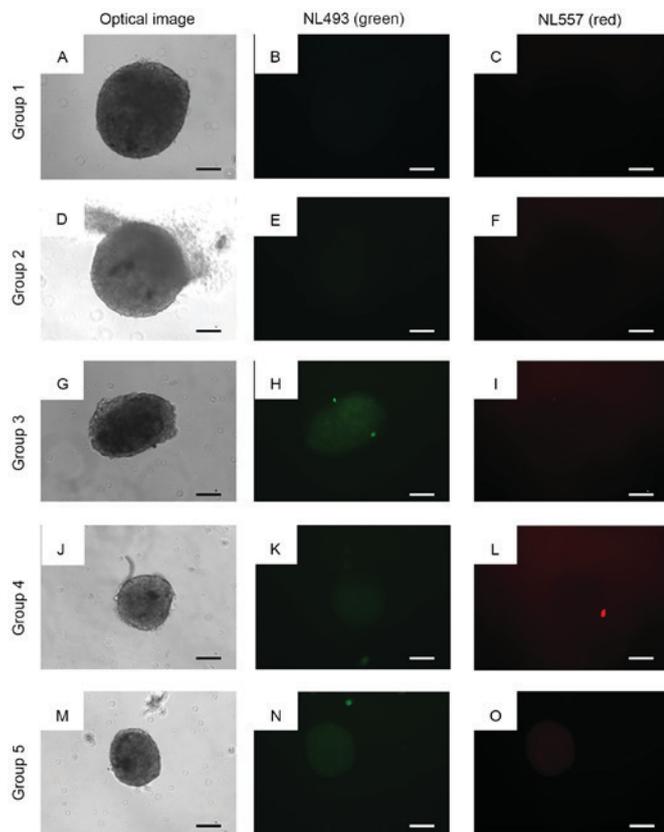


Figure 9. Evaluation of the maintenance of stemness with immunocytochemical staining at day 7. Cell spheroids were stained with NL493-conjugated SSEA-4 (green) and NL557-conjugated TRA-1-60(R) (red) antibodies. SSEA-4 and TRA-1-60(R) served as positive markers for stem cells. Immunohistochemical images of groups (A-C) 1, (D-F) 2, (G-I) 3, (J-L) 4 and (M-O) 5. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

groups 2 and 3 at day 1, when compared with group 1 at day 1 ($P < 0.05$). The relative values of groups 1, 2, 3, 4 and 5 at day 3 were 175.4 ± 7.1 , 199.1 ± 4.2 , 187.4 ± 8.6 , 192.8 ± 10.6 and $202.8 \pm 14.6\%$, respectively, when group 1 at day 1 was considered 100% ($100.0 \pm 7.2\%$). The relative values of groups 1, 2, 3, 4 and 5 at day 5 were 178.5 ± 11.2 , 164.7 ± 7.8 , 166.6 ± 3.3 ,

164.5 ± 4.6 and $162.5 \pm 4.0\%$, respectively, when group 1 at day 1 was considered 100% ($100.0 \pm 7.2\%$). The relative values of groups 1, 2, 3, 4 and 5 at day 7 were 156.8 ± 3.6 , 147.0 ± 6.6 , 151.5 ± 3.0 , 158.7 ± 4.2 and $141.2 \pm 5.8\%$, respectively, when group 1 at day 1 was considered 100% ($100.0 \pm 7.2\%$). Significant differences were noted between groups 1 and 5 at day 7 ($P < 0.05$).

Maintenance of stemness. Cell spheroids were stained with NL493-conjugated SSEA-4 (green) and NL557-conjugated TRA-1-60(R) (red) antibodies at day 7 (Fig. 9). In Group 1, spheroids were negative for the stem cell markers SSEA-4 and TRA-1-60(R).

Very low levels of fluorescence was seen in group 2 and intense green fluorescence was seen in groups 3-5. The green fluorescence was more intense in group 3 compared with that in groups 4 and 5. These results suggested that the co-culture cell spheroids contained undifferentiated human stem cells in groups 3-5.

Alkaline phosphatase activity assays. The results of the alkaline phosphatase activity assays are presented in Fig. 10. Statistically significant differences were observed between days 1 and 5 in groups 1, 2, 3 and 4, with a significant increase in absorbance level being demonstrated in each of these groups on day 5 compared with day 1 ($P < 0.05$). The absorbance values at 405 nm at day 5 for groups 1, 2, 3, 4 and 5 were 0.609 ± 0.031 , 0.603 ± 0.004 , 0.630 ± 0.024 , 0.568 ± 0.022 and 0.519 ± 0.026 , respectively. Additionally, group 1 demonstrated a significantly increased absorbance value at day 5 compared with group 5 ($P < 0.05$).

Mineralization assay. The results of the mineralization assay at day 7 are demonstrated in Fig. 11. Mineralized extracellular deposits were observed in each group.

Discussion

In the present study, cell spheroids were formed from human gingiva-derived stem cells and osteoprecursor cells using microwells. Results demonstrated that the shape, viability and stemness of the spheroids were maintained throughout the experimental period. The co-culture method may be applied for various applications due to enhanced functionality of the spheroids produced (9). Previous research has co-cultured primary pancreatic islets with hepatocytes for a three-dimensional model, and both types of cells appeared to support each other's functions (18). Three-dimensional co-culture of bone marrow-derived mesenchymal stem cells and eccrine sweat-gland cells in a gelatinous protein mixture has been demonstrated to promote the transdifferentiation of bone marrow-derived mesenchymal stem cells into potentially functional eccrine sweat-gland cells (19).

Previous research using co-cultures of osteoblasts with other cell types has demonstrated promising results in bone regeneration (20,21). A previous report have also demonstrated that osteoblasts and bone marrow-derived mesenchymal stem cells associate via gap junctions and that gap junction-mediated signaling induces histone acetylation that leads to elevated transcription of genes encoding alkaline phosphatase and

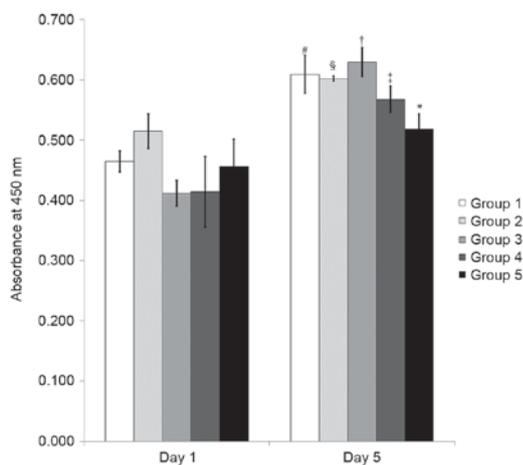


Figure 10. Alkaline phosphatase activity of spheroids on days 1 and 5. Data are presented as the mean \pm standard deviation. ^{*}P<0.05 vs. group 1 at day 5; [#]P<0.05 vs. group 1 at day 1; [§]P<0.05 vs. group 2 at day 1; [†]P<0.05 vs. group 3 at day 1; [‡]P<0.05 vs. group 4 at day 1; [¶]P<0.05 vs. group 5 at day 1.

bone sialoprotein in bone marrow-derived mesenchymal stem cells (22). Co-culture of human adipose-derived stem cells and human osteoblasts using three-dimensional poly [(R)-3-hydroxybutyric acid] with bovine-derived hydroxyapatite served as a promising approach to facilitate osteogenic differentiation activity through direct cell-to-cell contact (21). Three-dimensional spheroids generated from human mesenchymal stem cells and endothelial progenitor cells have demonstrated a greater angiogenic effect compared with stem cell mono-culture (23). Tissue engineering techniques for the regeneration of large bone defects require sufficient vascularization of the applied constructs to ensure a sufficient supply of oxygen and nutrients, and the concept of co-culture strategies may be applied to promote angiogenesis for cell-based, tissue-engineered bone grafts (24).

The present study demonstrated that viability of the spheroids was maintained during the experimental period using polydimethylsiloxane-based concave micromolds. A cell-counting kit using a water-soluble tetrazolium salt test was applied for the viability test as it assesses mitochondrial dehydrogenase activity and is reported to be more sensitive and less toxic to the tested cells when compared with the MTT test (7,25). The results of the present study demonstrated that cell spheroids formed from a single or two cell types preserved viability and may have promoted proliferation in the three-dimensional environment. Previous reports have indicated that co-culture spheroids generated by aggregating different combinations of primary human osteoblasts and human dermal microvascular endothelial cells exhibit excellent properties of preserving viability and promoting proliferation and vascularization (26). It may be suggested that the co-culture technique may be used to generate functional units for tissue-engineering purposes.

In conclusion, cell spheroids from gingival cells and osteoprecursor cells were able to maintain shape, viability, stemness and osteogenic differentiation potential. This method may provide a promising strategy for stem-cell therapy as a co-culture model.

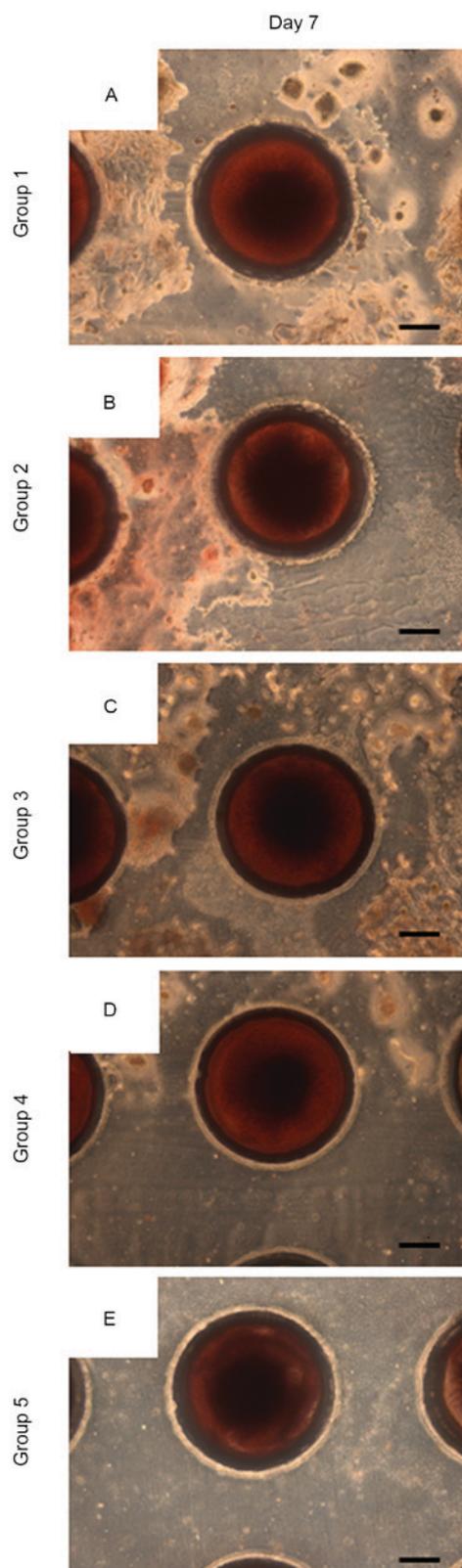


Figure 11. Alizarin Red S staining of spheroids on day 7. Magnification, $\times 100$; Scale bar, 200 μm .

Acknowledgements

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