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아스코르브산의 3차원 줄기세포 배양체에 대한 증식 및 분화 효과 분석

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ABSTRACT

The effects of ascorbic acid on the morphology and cell proliferation of three-dimensional stem cell spheroids

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Purpose: The effects of various concentrations of ascorbic acid on stem cell spheroids derived from intraoral areas are not known yet. Thus, the purpose of this study is to evaluate the effects of different concentrations of ascorbic acid on the morphology and cellular viability of stem cell spheroids derived from the gingival tissues.

Materials and Methods: Stem cells were plated onto silicon elastomer-based concave microwells and grown in the presence of ascorbic acid at concentrations ranging from 0.003% to 0.3%. The morphology of the cells was viewed under an inverted microscope at day 1, 2, 3 and 5. Qualitative live/dead assay and quantitative cellular viability using Cell Counting Kit-8 were performed on day 2 and day 5.

Results: Gingiva-derived stem cells formed spheroids irrespective of ascorbic acid concentration in silicon elastomer-based concave microwells. Increase in the diameter of spheroid were seen with higher concentrations of ascorbic acid. Higher cellular viability was seen in higher concentrations of ascorbic acid.

Conclusion: Within the experimental setting, the application of ascorbic acid on stem-cell spheroids produced an increase in the size and higher viability with higher dosage. It can be suggested ascorbic acid be applied with stem cell spheroids for tissue engineering purposes.

Key words : ascorbic acid; cell proliferation; cell survival; cellular spheroids; gingiva; stem cells

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I . INTRODUCTION

Ascorbic acid is the active component of vitamin C, and it is reported to be a naturally occurring organic compound with antioxidant properties¹⁻²). Ascorbic acid is reported to be an important co-substrate of a large class of enzymes and regulates gene expression by interacting with important transcription factors³). It is shown to play an important role in all stressful conditions linked to inflammatory processes and involve immunity³).

Ascorbic acid is shown to play a role in cell proliferation. A new class of antiproliferative molecules were suggested to come from ascorbic acid derivatives⁴). High concentrations of L-ascorbic acid specifically inhibit the growth of human leukemic cells via downregulation of hypoxia-inducible factor-1 α transcription⁵). Ascorbic acid and retinoic acid acted synergistically in inhibiting human breast cancer cell proliferation⁶). It was shown ascorbic acid may involve the expression of genes that induce differentiation and block proliferation and the up-regulation of antioxidant enzymes and proteins involved in apoptosis, cell cycle regulation and DNA repair⁶⁻⁷).

Ascorbic acid is shown to be associated with the differentiation of the tested cells^{2, 8, 9}). Ascorbic acid is an inhibitor of pre-adipocyte cell line differentiation with a dose-dependent effect²). Conversely, ascorbic acid was known to favor the expression of the osteoblastic phenotype in several bone cell systems⁸). The effects of ascorbic acid on pre-osteoblast gene

expression were tested, and ascorbic acid was shown to be essential for the proliferation and differentiation of preosteoblast⁹). Three-dimensional culture systems have been applied for the evaluation of the effects of the applied agents and they have various advantages over two-dimensional culture systems¹⁰⁻¹²). Cells in three-dimensional cultures can grow in all directions, mimicking the characteristics in vivo tissue¹⁰). The effects of various concentrations of ascorbic acid on stem cell spheroids derived from intraoral areas are not known yet. Thus, the purpose of this study is to evaluate the effects of different concentrations of ascorbic acid on the morphology and cellular viability of stem cell spheroids derived from the gingival tissues.

II . MATERIALS AND METHODS

1) Isolation and culture of human gingiva-derived stem cells

Gingival tissues were collected from healthy patients undergoing clinical crown lengthening procedures. The design of the study was reviewed and approved by the Institutional Review Board of the Catholic University of Korea's College of Medicine(no. KC11SIS I0348). Informed consent was obtained from all patients according to the Act on Legal Codes for Biomedical Ethics and Safety and the Declaration of Helsinki. Human gingiva-derived stem cells were isolated and cultivated following the protocol published in the author's previous study¹³).

The gingival tissues were collected and contained in sterile phosphate-buffered saline(PBS; Welgene, Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea) that included 100 U/mL penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA) at 4°C. The tissues were de-epithelialized, separated into 1-2 mm² fragments, digested in 0.2 μm filtered and modified in a media containing dispase(1 mg/ml; Sigma-Aldrich Co.) and collagenase type IV(2 mg/ml; Sigma-Aldrich Co.). The cell suspension was filtered with a 70 μm cell strainer(Falcon, BD Biosciences, Franklin Lakes, NJ, USA), and the cells were incubated at 37°C in a humidified incubator with 5% CO₂. After 24 hours, the non-adherent cells were washed with phosphate-buffered saline(Welgene, Daegu, South Korea). Fresh media was replaced every two to three days.

2) Formation of spheres and evaluation of cellular morphology

Cells were plated onto the silicon elastomer-based concave microwells(H389600, StemFIT 3D; MicroFIT, Seongnam, Korea) with 600 μm at a density of 4 x 10⁵ cells/each well and cultured in the media (alpha-minimal essential medium(α -MEM, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco), 200 mM L-glutamine(Sigma-Aldrich Co.), 10 mM of ascorbic acid 2-phosphate(Sigma-Aldrich)) in the presence of ascorbic acid(Sigma-Aldrich Co.) at final concentrations of 0.003%(x 1), 0.03%(x 10), and

0.3%(x 100) (Fig. 1). On day 2, 3 and 5, inverted microscopy(CKX41SF, Olympus Corporation, Tokyo, Japan) was used to evaluate the morphology of the tested stem cells.

3) Determination of cell viability

Spheroid viability was qualitatively analyzed by the Live/Dead Kit assay(Molecular Probes, Eugene, OR, USA). The assay is based on the principle that the activity of intracellular esterase causes non-fluorescent, cell-permeant calcein AM to become intensely fluorescent, giving the viable spheroids an intense, uniform, green fluorescence. Ethidium homodimer enters the damaged cell membrane and then binds to nucleic acids, thereby producing a red fluorescence in the dead cells.

The spheroids were washed twice with PBS, followed by suspension in 1 mL of α -MEM (Gibco) containing 2 μl of 50mM calcein acetoxymethyl ester working solution and 4 μl of the 2 mM ethidium homodimer-1 for 15 min at room temperature. In this way, the spheroids were stained with calcein acetoxymethyl ester and ethidium homodimer-1 and spheroids were observed under a fluorescence microscope (Axiovert 200; Zeiss, Germany) and confocal laser microscope(LSM800 w/Airyscan, Carl Zeiss, Germany) at day 2 and 5.

4) Determination of cell viability

The cell viability analysis was performed on day 1, 2 and 5. Tetrazolium monosodium

salt(Cell Counting Kit-8; CCK-8, Dojindo, Tokyo, Japan) was added to the culture, and the cells were incubated for 2 h at 37°C. The spectrophotometric absorbance at 450 nm was measured for the evaluation of formazan product using a microplate reader(BioTek, Winooski, VT, USA). The tests were performed three times.

5) Statistical analysis

The data were shown as means \pm standard deviations of the experiments. A test of normality was performed, and a one-way analysis of variance with post hoc Tukey's test was performed to determine the differences between the groups using a commercially available program(SPSS 12 for Windows, SPSS Inc., Chicago, IL, USA). The level of significance was considered 0.05.

III. RESULTS

1) Evaluation of cell morphology and cellular viability

Gingiva-derived stem cells formed spheroids irrespective of ascorbic acid concentration in silicon elastomer-based concave microwells(Fig. 1). The morphology of the stem cell spheroids at day 1 is shown in Figures 2A-2C. The morphology of the spheroids at day 2 was similar to that of day 1(Figs. 2D-2F). There were no significant changes in the morphology with the longer incubation time(Figs. 2G-2L).

2) Qualitative determination of cell viability

The results determined by fluorescent microscope are shown in Figure 3. Most of the cells in the spheroids emitted green fluorescence, and the morphology was round without

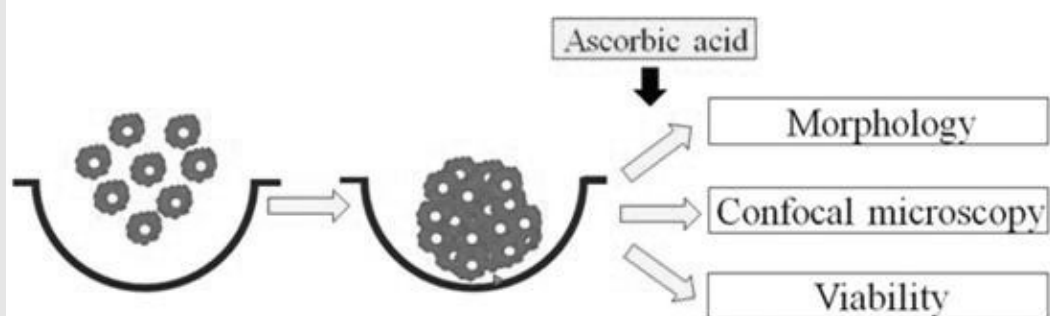


Fig. 1. Schematic illustration of stem cell spheroid fabrication with gingival-derived stem cells with different ascorbic concentrations.

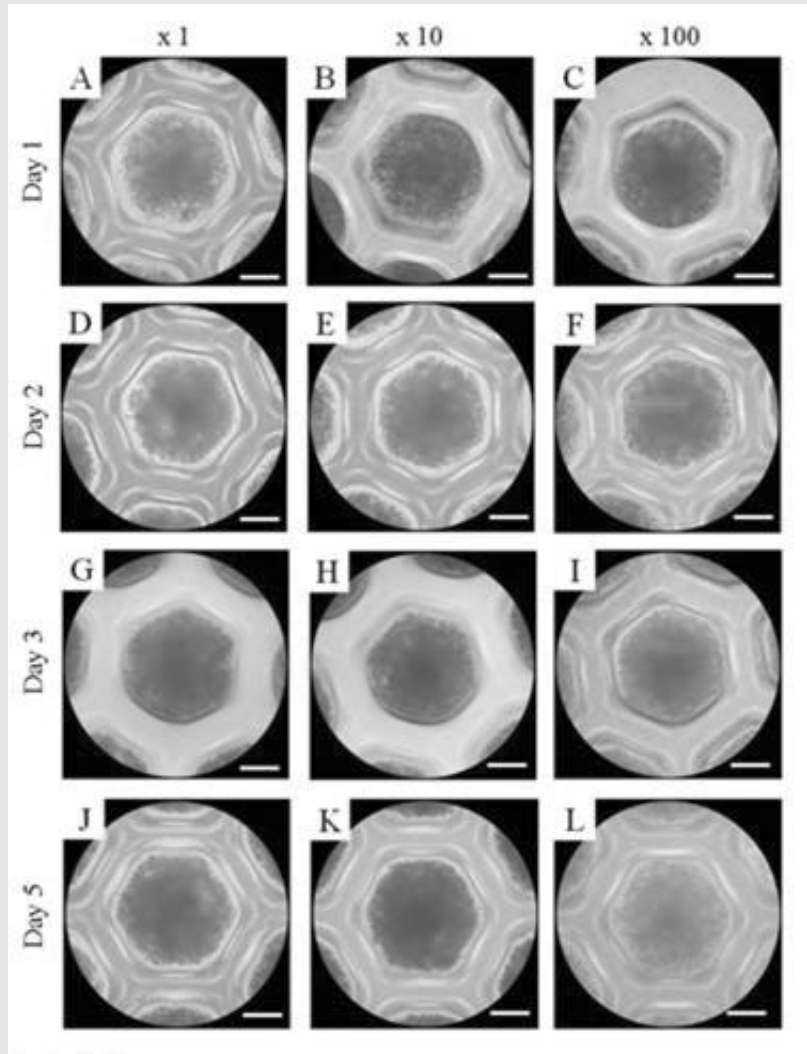


Fig. 2. The morphology of the stem cell spheroids at day 1, 2, 3 and 5. The scale bar indicates 200 μ m.

- (A) Group x 1 at day 1 (original magnification x 200).
- (B) Group x 10 at day 1 (original magnification x 200).
- (C) Group x 100 at day 1 (original magnification x 200).
- (D) Group x 1 at day 2 (original magnification x 200).
- (E) Group x 10 at day 2 (original magnification x 200).
- (F) Group x 100 at day 2 (original magnification x 200).
- (G) Group x 1 at day 3 (original magnification x 200).
- (H) Group x 10 at day 3 (original magnification x 200).
- (I) Group x 100 at day 3 (original magnification x 200).
- (J) Group x 1 at day 5 (original magnification x 200).
- (K) Group x 10 at day 5 (original magnification x 200).
- (L) Group x 100 at day 5 (original magnification x 200).

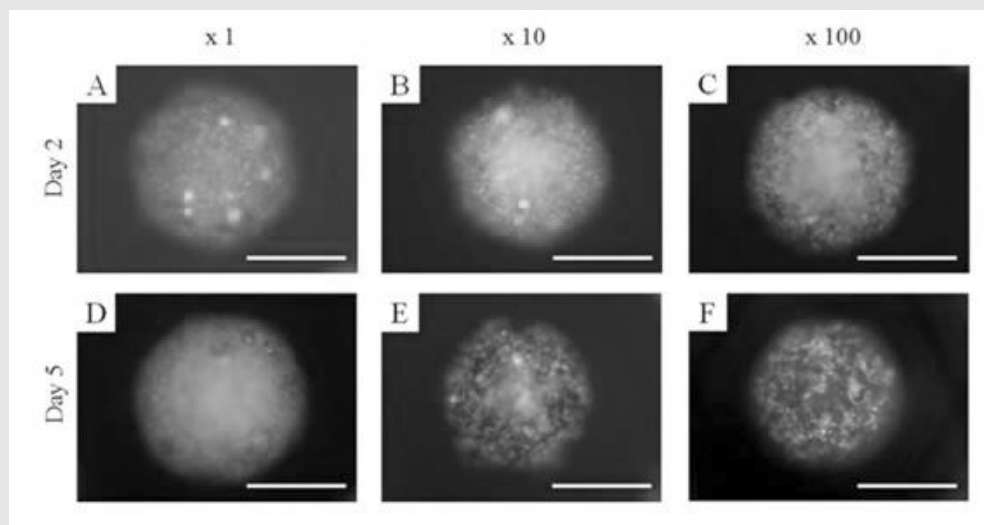


Fig. 3. Live/dead cell image of spheroids at day 2 and day 5 under fluorescent microscope. The scale bar indicates 500 μm .

- (A) Group x 1 at day 2 (original magnification x 100).
 (B) Group x 10 at day 2 (original magnification x 100).
 (C) Group x 100 at day 2 (original magnification x 100).
 (D) Group x 1 at day 5 (original magnification x 100).
 (E) Group x 10 at day5 (original magnification x 100).
 (F) Group x 100 at day 5 (original magnification x 100).

significant changes on day 2 and day 5. The results obtained under confocal microscope is shown in Figures 4 and 5. The results reveal a clear image of live and dead assay. The green fluorescence showed more intense assay with a higher concentration of ascorbic acid on day 2. However, the red fluorescence was also higher with an increase of the ascorbic acid concentration. The similar trends were seen in the results at day 5.

3) Diameter of stem cell spheroids and quantitative analysis of cell viability

The stem cell spheroid diameter results are seen in Figure 6. The average spheroid diameters

of the x 1, x 10 and x 100 groups at day 2 were $615.7 \pm 147.4 \mu\text{m}$, $651.0 \pm 127.5 \mu\text{m}$, and $694.2 \pm 86.4 \mu\text{m}$, respectively. The diameters of the x 1, x 10 and x 100 groups at day 5 were $623.6 \pm 151.1 \mu\text{m}$, $631.6 \pm 134.9 \mu\text{m}$ and $690.7 \pm 86.4 \mu\text{m}$, respectively. There were increasing diameters with higher concentrations of ascorbic acid; however, this did not reach statistical significance ($P > 0.05$).

The results of cell viability using CCK-8 after culturing at day 1, 2, and 5 are shown in Figure 7. The viability values of the x 1, x 10 and x 100 groups at day 1 were 0.318 ± 0.019 , 0.300 ± 0.058 and 3.484 ± 0.303 , respectively ($P < 0.05$). The viability values of the x 1, x 10 and x 100 groups at day 2 were 0.350 ± 0.019 , 0.507

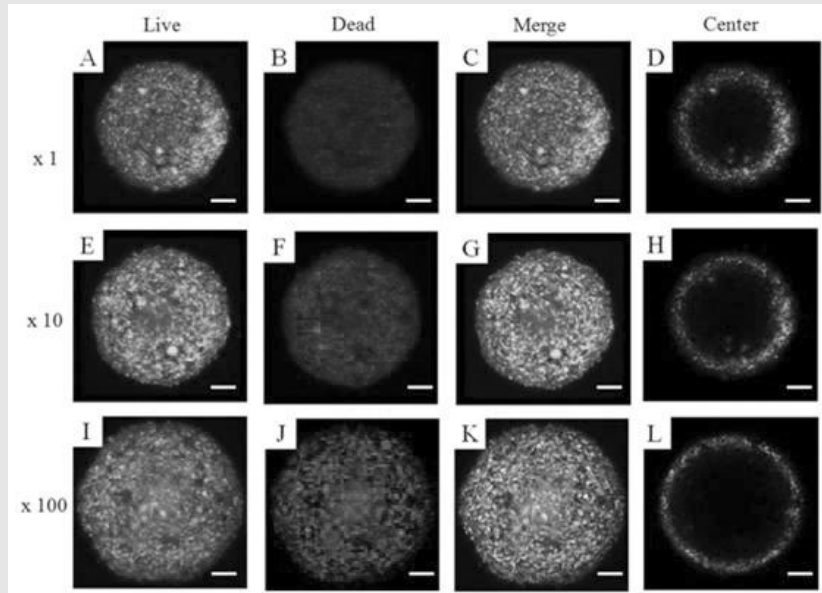


Fig. 4. Qualitative results of cellular viability at day 2 under confocal microscope. Live image, dead image, merged image and central images are provided. The scale bar indicates 100 μm .
 (A-D) Group x 1 (original magnification x 100).
 (E-H) Group x 10 (original magnification x 100).
 (I-L) Group x 100 (original magnification x 100).

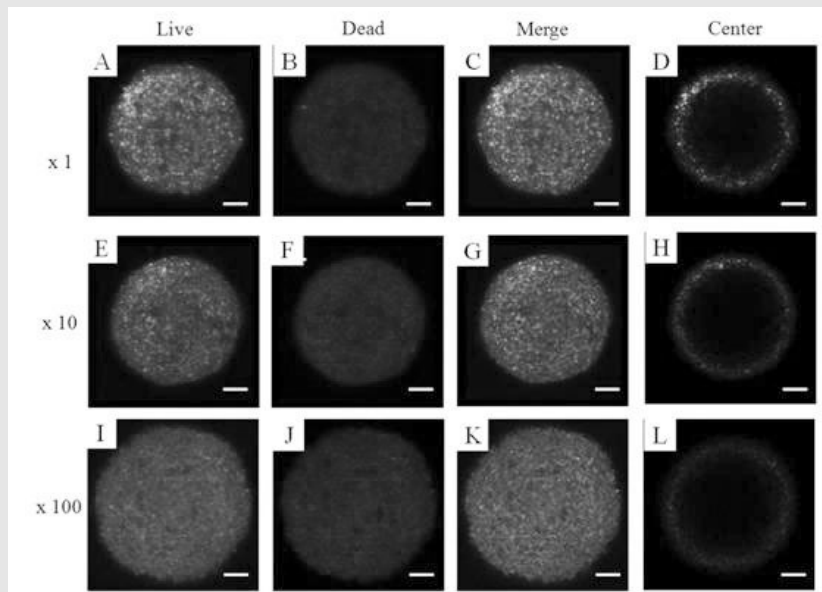


Fig. 5. Qualitative results of cellular viability at day 5 under confocal microscope. Live image, dead image, merged image and central images are provided. The scale bar indicates 100 μm .
 (A-D) Group x 1 (original magnification x 100).
 (E-H) Group x 10 (original magnification x 100).
 (I-L) Group x 100 (original magnification x 100).

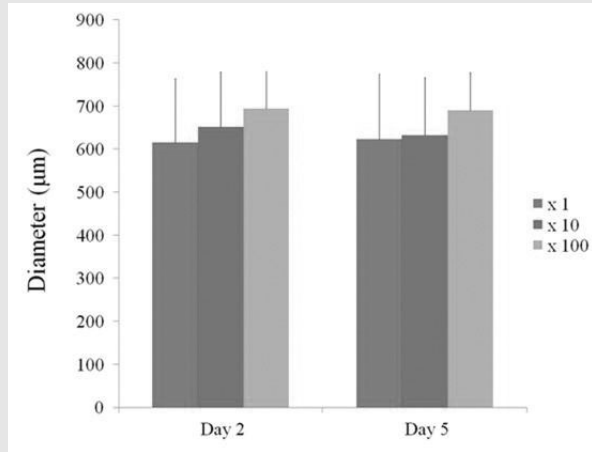


Fig. 6. The diameter of the spheroids at day 2 and day 5.

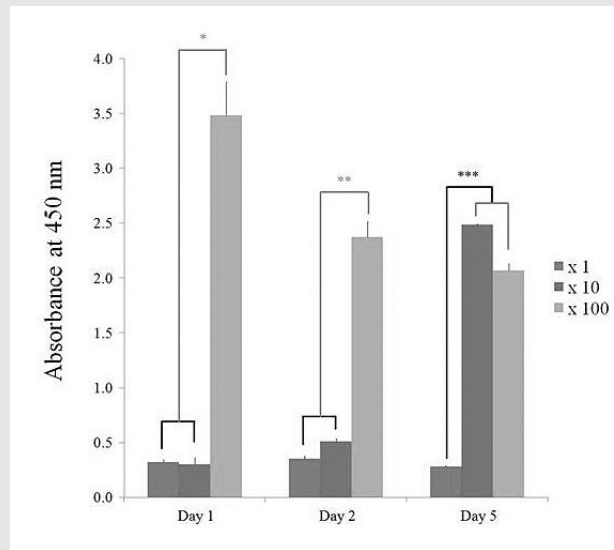


Fig. 7. Cellular viability on day 1, 2 and 5.

* Statistically significant differences were noted when compared with group x 1 and x 10 at day 1.
 ** Statistically significant differences were noted when compared with group x 1 and x 10 at day 2.
 *** Statistically significant differences were noted when compared with group x 1 at day 5.

± 0.025 and 2.374 ± 0.141 , respectively ($P < 0.05$). The results at day 5 were 0.276 ± 0.013 , 2.483 ± 0.006 and 2.067 ± 0.062 , respectively ($P < 0.05$).

IV. DISCUSSION

This report discusses the effects of different concentrations of ascorbic acid on the morphology and cellular viability of stem cell spheroids derived from the gingival tissues.

Ascorbic acid used in the test seemed to affect the morphology of spheroids stem cells. An increase in the diameter of the spheroids was noted with a higher concentration of ascorbic acid within the tested concentration and qualitative CCK-8 results showed higher mitochondrial activity with a higher concentration of ascorbic acid. In the previous report, the addition of ascorbic acid resulted in cell viability increase⁸). The dosage of ascorbic acid varies among different studies^{5, 8, 14, 15}). The dosage used in this study corresponds to 0.16 mM, 1.6 mM and 16 mM. Previous studies testing the effects of ascorbic acid on osteoblast cells used the concentration of 284 μM ⁸). In another report, the researchers suggested the recommended dosage of 50 μM for the osteogenic differentiation of bone marrow derived stem cells¹⁵). It should be noted the use of higher doses of ascorbic acid may yield a negative effect on cell viability⁵). However, there could be dose variations used to achieve the maximal effect, depending on the system model, the stage of differentiation of stem cells and the culturing time period^{14, 16}).

Many ways have been suggested for making the cell spheroids¹⁷⁻²⁰). The hanging drop method is one of the most widely used for the fabrication of three-dimensional spheroids²¹). Another bioreactor method-having a rotating platform-can be applied for cell spheroids¹⁸). In this report, the silicon elastomer-based concave microwells were used for the fabrication of stem cell spheroids. Microwell culture systems were suggested to produce spheroids in the defined

size with homogenous colonies²²). Microwells having a poly(ethylene glycol) surface were reported to generate retrievable cell colonies with controlled sizes and shapes with high viability²⁰).

Cells in spheroids show different characteristics over two-dimensional monolayer cultures¹¹). These spheroids are shown to interact with their surroundings in all three dimensions better than in two-dimension culture systems¹²). It has also been suggested three-dimensional cultures show higher stability and higher resemblance to human tissue, and this may lead to less use of animal models for the experiments¹¹). Moreover, cell spheroids, when applied in cell therapy, may have greater advantages. Stem cell spheroids are reported to show longer stemness maintenance when compared with two-dimensional cultures²³). Stem cell spheroids are shown to secrete significantly higher amounts of growth factors, including vascular endothelial growth factor A²⁴). These three-dimensional spheroids can be maintained without routine passaging and manipulation^{25, 26}). In another study, alkaline phosphatase activities were significantly higher in the three-dimensional cultures when compared with the two-dimensional cultures^{17, 27}). Additionally, these stem cell spheroids can be applied in regeneration medicine without the aid of a scaffold with a higher cell number^{22, 28}).

Various tissues can be used for the candidate of stem cells, including bone marrow, adipose tissue and peripheral blood^{29, 30}). Bone marrow is a reasonable source for stem cells, but the

procedure for obtaining stem cells may lead to higher pain and morbidity²⁹⁾. However, the intraoral area is a more attractive source for mesenchymal stem cells because such a procedure can be performed under local anesthesia³¹⁾. The gingival tissue can be obtained during routine daily practices with less limitations for the supply^{32, 33)}.

V. CONCLUSION

The application of ascorbic acid on stem-cell spheroids produced an increase in the size and higher viability with higher dosage within the experimental setting. It can be suggested ascorbic acid be applied with stem cell spheroids for tissue engineering purposes.

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