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Chapter 7

# Morphological Comparison of Stem Cells Using Two-Dimensional Culture and Spheroid Culture

Sae Kyung Min, Hyunjin Lee, Minji Kim and Jun-Beom Park

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**Abstract**

Mesenchymal stem cells are of great interest, especially in regeneration medicine. Mesenchymal stem cells have the ability to differentiate into several tissues including bone and fat. The stem cells can be obtained from various tissues including bone marrow, periosteum, gingiva, and tooth. Traditionally, two-dimensional culture has been applied for stem cell research. However, more recently, a three-dimensional model has been of great interest for studying the stem cells because it mimics the physiological conditions. Spheroid culture is one way of applying three-dimensional culture. This report describes the two-dimensional culture and spheroid culture and the morphological comparison will be performed between two-dimensional culture and spheroid culture.

**Keywords:** bone marrow, cellular spheroids, gingiva, organ culture techniques, stem cells

## 1. Introduction

Mesenchymal stem cells are stromal cells that can be differentiated into bone, cartilage, and fat cells [1]. These stem cells can be found in various tissues, including bone marrow and fat [2]. The ability to form cellular aggregations has been utilized in a three-dimensional model [3]. These three-dimensional structures using stem cells were reported to maintain cell survival and function and were applied for tissue engineering purposes [4]. Spheroid culture has recently been of interest, especially for regeneration purposes [5]. In detail, spheroid culture produces an increased secretion of cytokines, such as vascular endothelial growth factor and granulocyte

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colony stimulating factor, when compared with that from two-dimensional cultures [6]. The aim of this review was to describe the two- and three-dimensional cultures, and the morphological comparison will be performed between two-dimensional culture and spheroid culture.

## 2. Characteristics of stem cell research

Mesenchymal stem cells are characterized by the capability of osteogenic, adipogenic, and chondrogenic differentiation [7]. Previously, the stem cells derived from the periosteum and bone marrow of the jaw bone (mandible) and long bone (tibia) were compared in order to determine a suitable cell source [8]. A bone marrow-derived mesenchymal stem cell sheet with platelet-rich plasma could promote bone regeneration [9]. Bone marrow is an attractive source of stem cells, but gaining stem cells from bone marrow may produce greater pain and morbidity [10]. Stem cells can also be achieved intraorally, and gingiva may serve as a more feasible source for stem cells because obtaining gingival-derived stem cells can be done under local anesthesia with less pain and morbidity [11].

## 3. Morphological evaluation of two-dimensional stem cell culture

**Figure 1** shows morphology of the stem cells cultured in an alpha-minimal essential medium (α-MEM, Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum (Gibco), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA), 200 mM of L-glutamine (Sigma-Aldrich Co.), and 10 mM of ascorbic acid 2-phosphate (Sigma-Aldrich Co.) on Day 12. We plated stem cells at the seeding density of $1.3 \times 10^4$ cells/cm$^2$. The media were changed every 2–3 days, and cells were incubated in an incubator with 5% $CO_2$ and 95% $O_2$ at 37°C. The cells were observed under an inverted microscope (Leica DM IRM, Leica Microsystems, Wetzlar, Germany) (CKX41SF, Olympus Corporation, Tokyo, Japan), and the images were saved as JPGs.

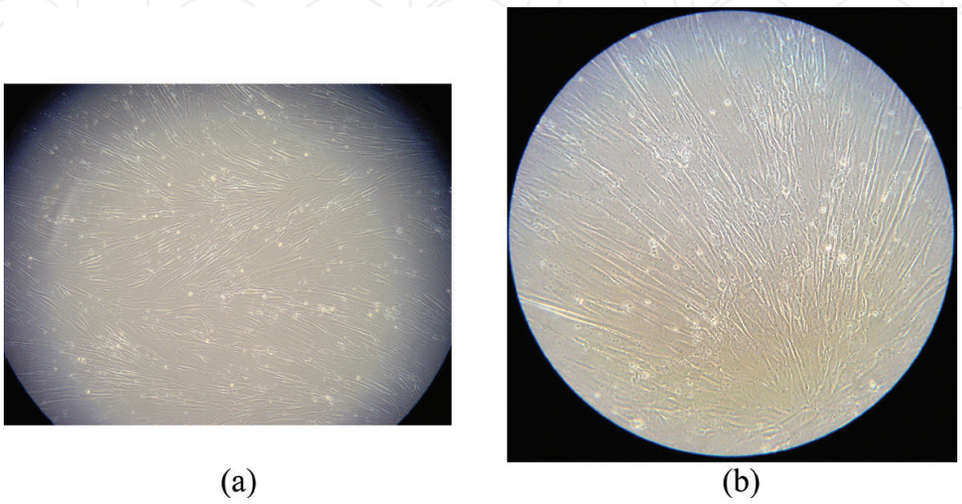



**Figure 1.** Morphology of the stem cells cultured in growth media on Day 12. (a) The view shows the cells with higher confluence (original magnification 200×) and (b) higher magnification (original magnification 400×).

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**Figure 2** shows morphology of the stem cells cultured in adipogenic media (STEMPRO® Adipogenesis Differentiation Kit, Gibco, Grand Island, NY, USA). The cells were supplied with adipogenic induction medium and adipogenic maintenance medium alternately. The cells' morphology was viewed under an inverted microscope (Leica DM IRM). **Figure 3** shows morphology of the stem cells cultured in adipogenic media for a longer period of 13 days.

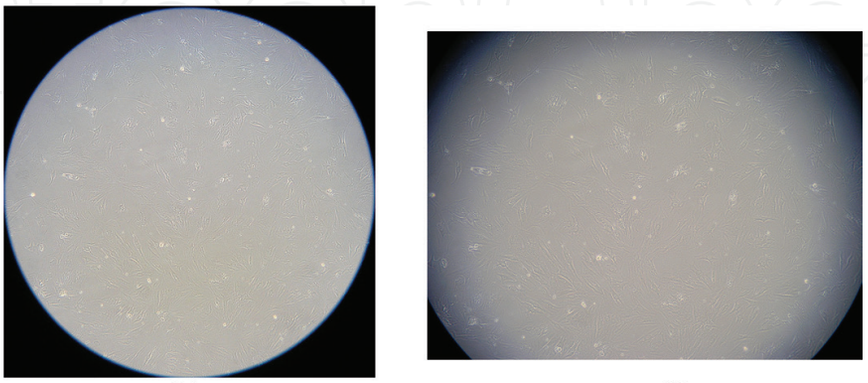



**Figure 2.** Morphology of the stem cells cultured in adipogenic media on Day 5. (a) The morphology of the cells at low magnification (original magnification 100×) and (b) higher magnification (original magnification 200×).

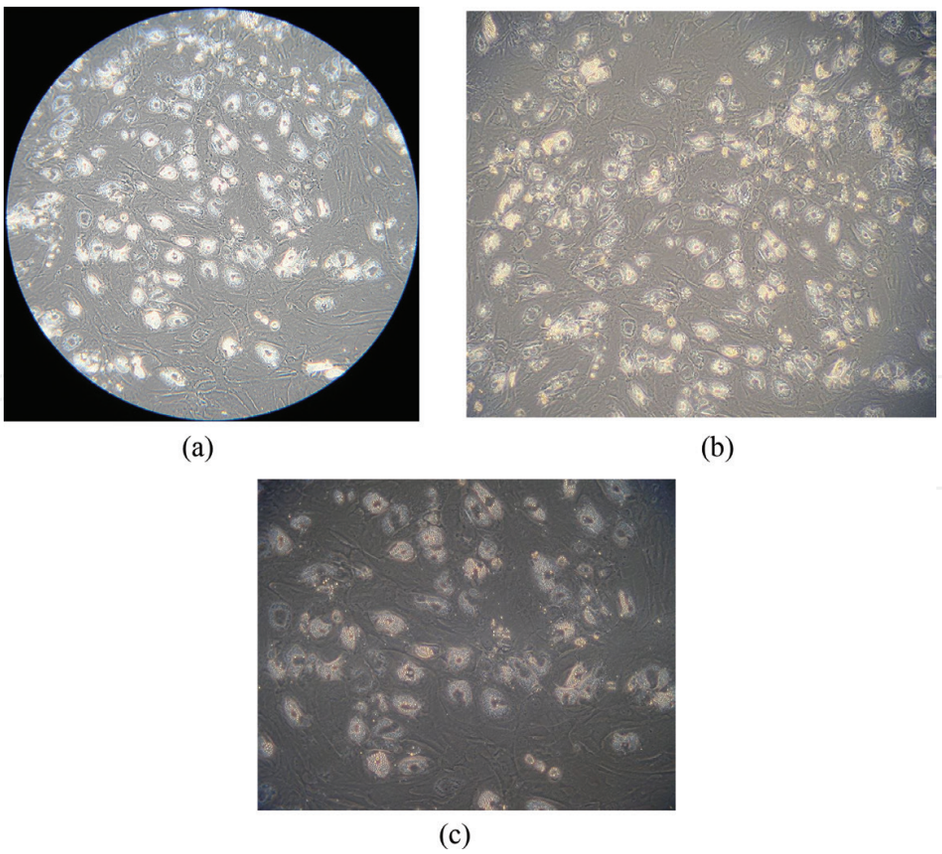



**Figure 3.** Morphology of the stem cells cultured in adipogenic media on Day 13. (a) The morphology of the cells at low magnification (original magnification 100×), (b) higher magnification shows that cells have a ghost-like feature (original magnification 100×) and (c) more distinct feature of abiogenesis is shown (original magnification 200×).

**Figure 4** shows that the morphology of stem cells treated with a chemotherapeutic agent of doxorubicin at 10 μg/mL on Days 1, 3, 5, and 7. A cell viability analysis of the stem cells was performed on Days 1, 3, 5, and 7. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] (CCK-8; Dojindo, Tokyo, Japan) was added to the cultures, and the spheres were incubated for 1 h at 37°C. Viable cells were identified by the assay, which relies on the ability of mitochondrial dehydrogenases to oxidize

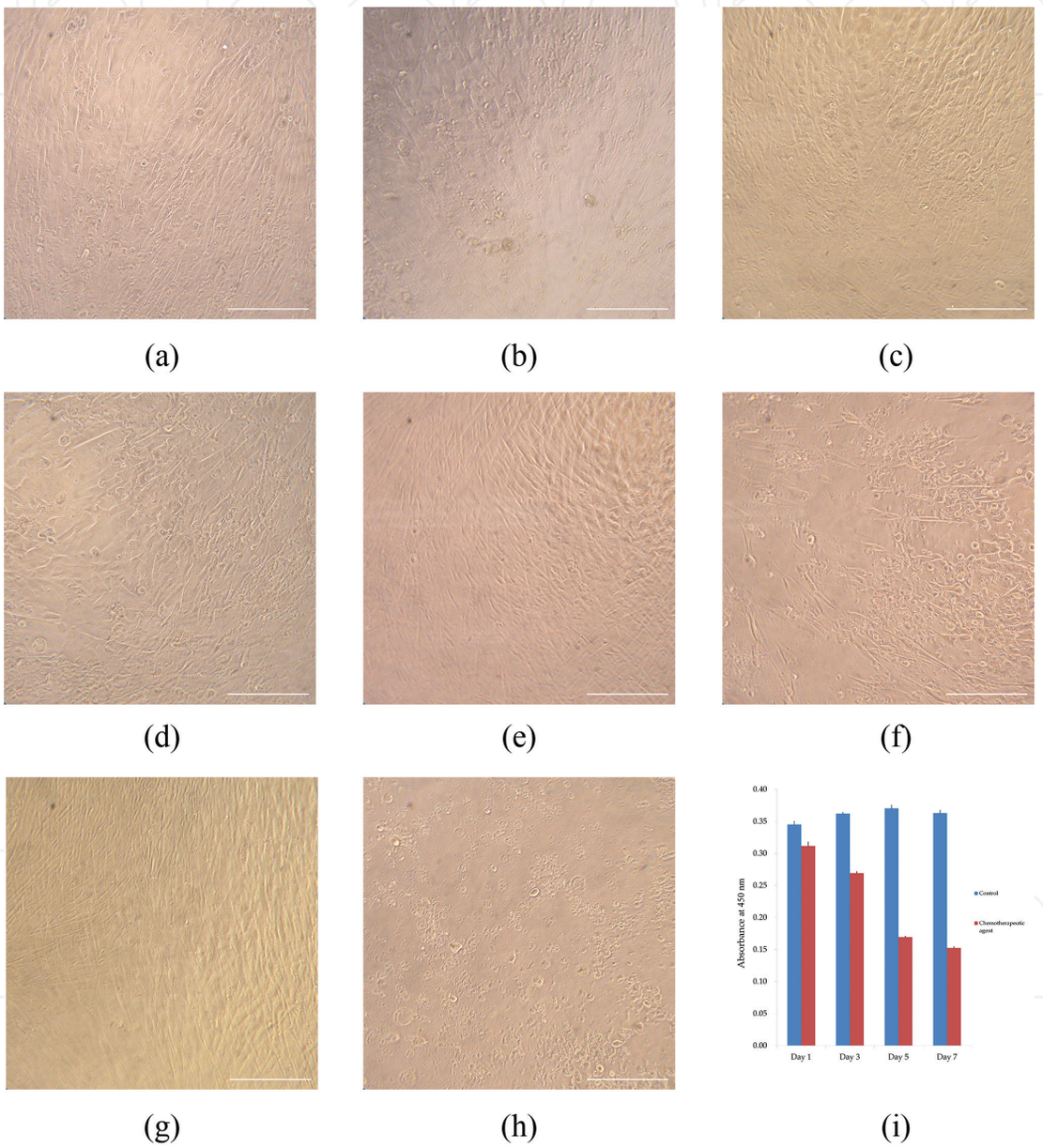



**Figure 4.** Morphology of the stem cells in growth media: (a) untreated group on Day 1 (original magnification 200×); (b) chemotherapeutic group of doxorubicin at 10 μg/mL on Day 1 (original magnification 200×); (c) untreated group on Day 3 (original magnification 200×); (d) chemotherapeutic group of doxorubicin at 10 μg/mL on Day 3 (original magnification 200×); (e) untreated group on Day 5 (original magnification 200×); (f) chemotherapeutic group of doxorubicin at 10 μg/mL on Day 5 (original magnification 200×); (g) untreated group on Day 7 (original magnification 200×); (h) chemotherapeutic group of doxorubicin at 10 μg/mL on Day 7 (original magnification 200×); and (i) cellular viability of the stem cells on Days 1, 3, 5, and 7 using CCK-8. The bar indicates 200 μm.

WST-8 into a formazan product. The spectrophotometric absorbance of the samples was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). No significant morphological change of the stem cells cultured in growth media was observed after the addition of the chemotherapeutic agent on Day 1. More significant changes in the morphology of the stem cells were seen in the chemotherapeutic agent-treated group with longer incubation. A decrease in cellular viability was noted with treatment of the chemotherapeutic agent.

## 4. Titanium research

**Figure 5** shows the morphology of the stem cell culture on modified titanium discs. Machined titanium discs measuring 10 mm in diameter and 2 mm in thickness were used. The stem cells were plated at a density of $1.0 \times 10^5$ cells/well on 24-well plates containing titanium discs and cultured. Each implant disc was fixed with 4% paraformaldehyde at room temperature for 30 min. Permeabilization was performed with 0.1% Triton X-100/Dulbecco's phosphate-buffered saline for 2 min and blocking solution consisting of 0.2 μm filtered 1% bovine serum albumin/Dulbecco's phosphate-buffered saline for 30 min. Actin filaments were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR), and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole. The cells were observed using a confocal laser microscope (LSM5 Pascal, Zeiss, Jena, Germany) at a magnification of 200×. The cells attached to the titanium discs showed well-organized actin cytoskeletons with blue nuclei with confocal microscopy.

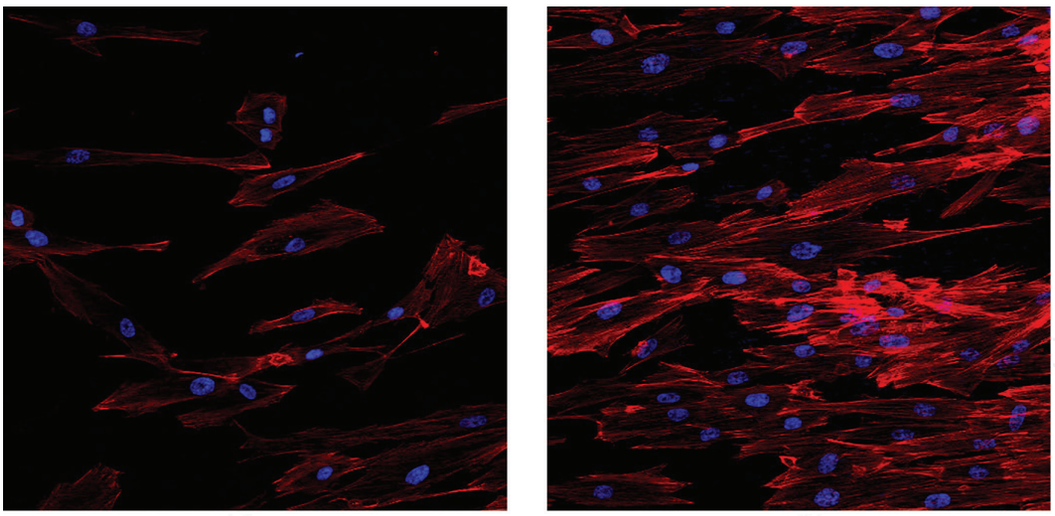



**Figure 5.** The morphology of stem cells culture on modified titanium discs. (a) Disc with limited number of cells (objective lens 20×) and (b) disc with higher number of cells (objective lens 20×).

## 5. Three-dimensional culture

In more recent years, three-dimensional cell culture methods have been widely applied and are regarded to have high importance in evaluating the biological processes [12]. Three-dimensional

culture systems may simulate the intercellular interactions in regulation of stem cell self-renewal and differentiation [13]. It was shown that a three-dimensional culture enhanced the production of extracellular matrix-related genes when compared with two-dimensional monolayer culture [12].

## 6. Spheroid culture

Spheroid cultures have an advantage of making three-dimensional cell aggregates without using exogenous materials [14]. Three-dimensional cell spheroids can be fabricated using various methods including silicon elastomer-based concave microwells and the hanging drop method [14]. **Figure 6** shows the morphology of cell spheroids cultured in growth media. Gingival tissues were collected from the healthy participants visiting the Department of Periodontics, Seoul St. Mary's Hospital. The Institutional Review Board of Seoul St. Mary's Hospital College of Medicine, Catholic University of Korea, Seoul, Republic of Korea, approved the study, and informed consent from the study participants was obtained. All the methods used in this study were performed in accordance with the relevant guidelines and regulations. In short, gingivae were de-epithelialized, minced into 1–2 $mm^2$ fragments, and digested in an alpha-modified minimal essential medium (α-MEM, Gibco, Grand Island, NY, USA) containing collagenase IV (2 mg/mL, Sigma-Aldrich Co., St. Louis, MO, USA) and dispase (1 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% $CO_2$. After 24 h, the non-adherent cells were washed with phosphate-buffered saline (Welgene, Daegu, South Korea). Fresh media was replaced every 2–3 days. Stem cell spheroids were formed in the silicon elastomer-based concave microwells (H389600, StemFIT 3D; MicroFIT, Seongnam, Korea) with 600 μm diameters. Gingiva-derived stem cells and bone marrow-derived stem cells in the amount of $1 \times 10^6$ were seeded and subsequently cultured to investigate cellular behavior. Inverted microscopy (CKX41SF, Olympus Corporation, Tokyo, Japan) was used to evaluate the morphology of the tested stem cells. Spheroids were well formed in silicon elastomer-based concave microwells using gingiva-derived stem cells.

Secretion of growth factors may differ between two-dimensional cultures and three-dimensional cell spheroids [6]. In a previous report, two- and three-dimensional systems were used for the determination of secreted human vascular endothelial growth factor using a commercially available kit (Quantikine® ELISA, R&D Systems, Inc., Minneapolis, MN, USA) [6]. The osteogenic differentiation of gingiva-derived stem cells grown on culture plates or in stem cell spheroids were evaluated by comparing two- and three-dimensional cultures, and the results indicated that gingiva-derived stem cell spheroids exhibit an increased osteogenic potential compared with stem cells from two-dimensional culture [11]. The co-culture of various cells including stem cells and primary cells can be done at various ratios [5]. Enhanced osteogenic differentiation may be achieved by applying the co-culture of stem cells and endothelial cells [15].

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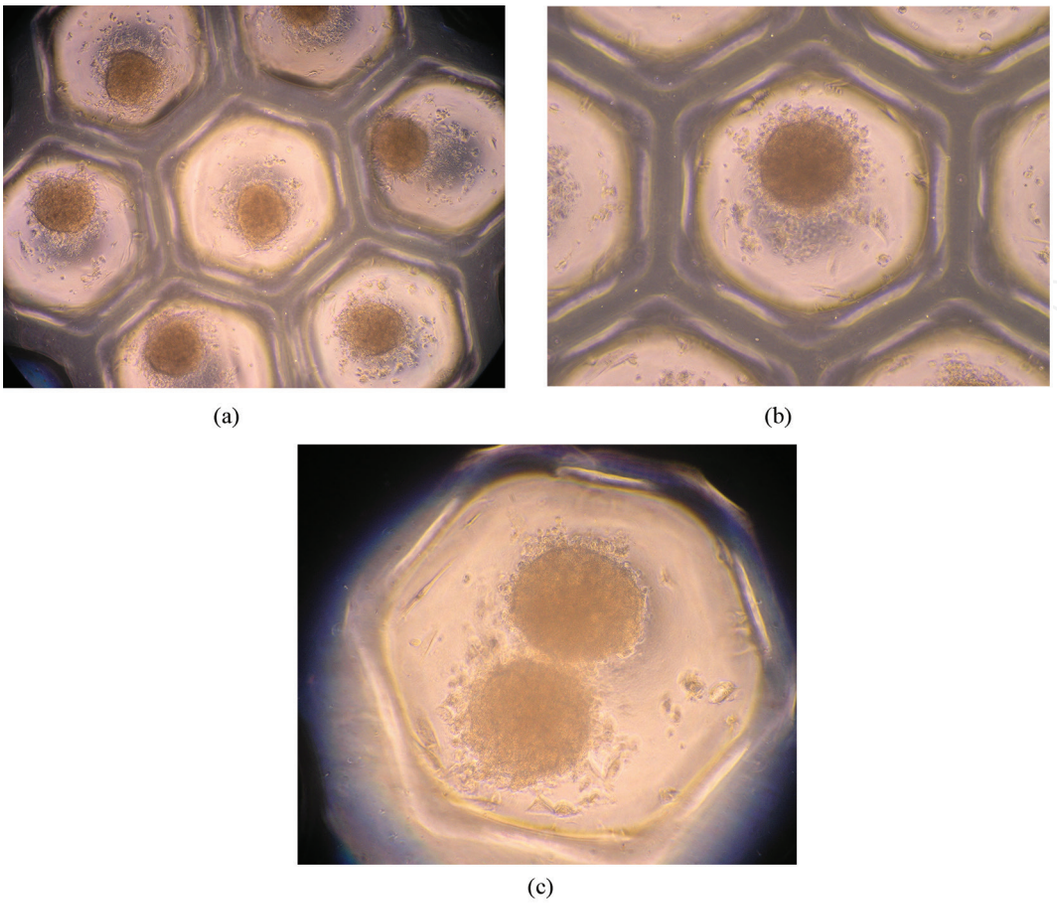



**Figure 6.** The morphology of the stem cell spheroids on Day 5. (a) The morphology of the stem cell spheroids at low magnification (original magnification 100×), (b) higher magnification (original magnification 200×) and (c) the number of stem cell spheroids in the well is more than one (original magnification 200×).

## 7. Conclusions

This report describes the two-dimensional culture and spheroid culture, and the morphological comparison will be performed between two-dimensional culture and spheroid culture. Spheroid cultures have an advantage of making three-dimensional cell aggregates without using exogenous materials, and this approach will be more widely applied as one of the three-dimensional cell culture methods to evaluate the biological processes.

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## Conflict of interest

The authors confirm that they have no competing interests.

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