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Effect of Culture Conditions on Colony-Forming Ability of Stem Cells from Human Exfoliated Deciduous Teeth

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Abstract

Stem cells from human exfoliated deciduous teeth (SHED) are originally referred to as fibroblastoid-colony-forming-cells because one of their characteristics is related to proliferation and differentiation potential. In this study, *we developed an efficient protocol for colony-forming-unit (CFU) assay for SHED.* The culture conditions including culturing time, seeding cell density and modifications of media supplements were optimized. The CFUs were determined on the proportion of colony types, sizes and the numbers of colony. Isolated SHED expressed CD44, CD73, CD105 and showed multipotent ability. Higher yield was obtained when SHED was cultured at the density of 500 cells per well of a 6-well plate for 14 days in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). The number and size of CFU was increased with the increase of FBS concentration in which the transit-amplifying cell (TAC) colonies were the most abundant, followed by the stem cell (SC) and differentiated cell (DC) colonies, respectively. In DMEM with 20% FBS and 100 μM L-ascorbic acid-2-phosphate, the CFU rate was highest with increasing the proportion of SC colonies. The improvement of the culture conditions described herein could be adopted to easily acquire more functional SHED for applying in cell therapy.

Introduction

A dental pulp tissue appears to be an excellent source of adult mesenchymal stem cells (MSCs). Clinically, interesting populations of pulp cells have also been isolated from deciduous teeth [1]. The stem cells from human exfoliated deciduous teeth (SHED) with self-renewal and multilineage differentiation capacities have been found to grow more rapidly than those from other sources. It is believed that this is because they may be less mature than other stem cells found in the body. Moreover, the stem cells from exfoliating deciduous teeth are readily available providing that they are stored properly until they are

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needed later in life and are used with a minimally invasive process that results in minimal trauma [2].

One of the most prominent properties of MSCs is their ability to generate fibroblastoid colony-forming cells, termed colony forming unit-fibroblasts (CFU-Fs) after they are plated at low density [3, 4]. The CFU-Fs are representative of the long term self-renewable and rapid proliferation properties in MSCs [5]. The efficiency with which they form colonies still remains an important assay to determine the quality of cell preparations. From several studies, there are generally three types of colonies, called holoclones, meroclones from transit-amplifying cells and paraclones [6-8]. The holoclones are large, contain 20,000–50,000 cells and consist mainly of small cells. The meroclones also have considerable proliferative capacity but are not as large as holoclones and have a greater range of cell size. When meroclones are subcloned, more than 5% but less than 100% of the colonies produced are terminal. The paraclones are small, highly irregular colonies with a short lifespan (less than 15 cell generations), consist mainly of large cells and do not form colonies on subcloning [6-8]. The holoclones are likely to be derived from stem cells (SC), meroclones from transit-amplifying cells (TAC) and paraclones from late stage precursors which quickly differentiate to become terminal or terminally differentiated cell colonies (DC) [6-8]. On the basis of isolation and expansion protocols, it is expected that the CFU-Fs originated from MSCs, as a result of clonal evolution, would rapidly become fast growing with a high clonogenic capacity. However, they actually have different proliferative and differentiation potentials which may be either subtle or significant [9, 10]. There is large variation in CFU-Fs among the cell lines [11]. Nevertheless, the CFU-Fs and the growth rate of most individual cell lines seem to be stable over many generations. Isolation facilities and protocols, including medium, plastic, seeding density, growth factors, and chemicals, influence the expansion, differentiation, and immunogenic properties of MSCs [12]. The optimal conditions that influence the CFU-Fs in SHED and the effects on the proliferative capacity of individual colonies have not been clearly studied. In order to increase the efficiency of colony-forming unit fibroblast formation in SHED culture, we examine the effects of culturing time, seeding cell density and modification of media supplements, serum and ascorbic substrates, on colony morphology and production colonies in an attempt to better optimize the isolation and generation of CFU-Fs from SHED.

Materials and Methods

Tooth Sample Collection

Human exfoliated deciduous teeth were acquired as discarded biological samples from children (5-9 years of age) at Faculty of Dentistry, Mahidol University following the approval of the ethical committee of the Faculty of Dentistry, Mahidol University (IRB 2014/044.2710).

Isolation and Culturing of SHED

After disinfection of the tooth surface, the teeth were mechanically fractured and the dental pulps were gently isolated with forceps. The pulp tissue was rinsed in a growth medium [Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) from Biochrom GmbH, Berlin, Germany or from Sigma St. Louis, MO, USA and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA)], after which it was cut into small pieces of 1–2 mm^3. The small tissue pieces were cultured in 25 mm^2 culture flasks in growth medium at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days and the cell cultures were monitored regularly with an inverted phase-contrast microscope (Nikon, Eclipse TS100, Tokyo, Japan). When reaching 70%–80% confluence, cells were harvested by using 0.05% Trypsin/EDTA (Sigma) and sub-cultured for further experiments. The cells between passage 3 and 5 were used. For each assay, the stem cells of at least three different donors were used.

Immunophenotypic Characterization of SHED

For the cell surface antigen analysis, SHED were harvested, washed in cold PBS supplemented with

0.5% Bovine Serum Albumin (BSA, Sigma), and aliquots of 5 x 10^5 cells were labeled (for 30 min in the dark at 4°C) with monoclonal antibodies specific for human markers associated with mesenchymal and hematopoietic lineages. Namely, mouse anti-human antibodies against the following antigens were used: CD34 (FITC conjugated), CD44 (FITC conjugated), CD73 (APC conjugated), and CD105 (PE conjugated) (all purchased from BioLegend, San Diego, CA, USA). To determine the level of nonspecific binding, fluorochrome conjugated isotype control antibodies were used. Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA).

Differentiation

After 28 days in complete commercial medium (HyClone™ AdvanceSTEM™ Osteogenic Differentiation Kit, Hyclone) for osteogenesis, calcium depositions were demonstrated in the extracellular matrix. The cells were fixed in 10% paraformaldehyde for 15 min at room temperature (RT). Cultures were then stained with 40 mM Alizarin Red S solution (Sigma), pH 4.1, for 20 min at RT, followed by rinsing three times with deionized water.

After 28 days in complete medium (HyClone™ AdvanceSTEM™ Adipogenic Differentiation Kit, Hyclone) for adipogenesis, cells were fixed in 4% paraformaldehyde for 15 min at RT and stained with a fresh 0.35% Oil Red O solution for 10 min, followed by washing twice with deionized water. The differentiated cells were observed and imaged using an inverted microscope.

After 21 days in D-MEM, 10% FBS, 10 ng/ml TGF-β1 (sigma), 100 nmol/L dexamethasone (sigma), 6.25 µg/ml insulin (sigma), 110 mg/L sodium pyruvate (sigma) and 1% penicillin/streptomycin for chondrocyte differentiation, proteoglycans of differentiated cells were examined by staining with Alcian blue (Sigma), pH 2.5, for 20 min at RT, followed by three washes in tap water to remove unspecific binding of the dye. The differentiated cells were observed using an inverted microscope and images were taken.

After 14 days in DMEM/F12 (Gibco), 20 ng/ml neurotrophins brain-derived neurotrophic factor (BDNF), 20 ng/mL neurotrophin-3 (NT-3), 20 ng/mL glial cell-derived neurotrophic factor (GDNF) (all from Sigma), supplemented with 2% N-2 (Gibco), 2% B-27 supplement, 100 U/mL Penicillin, and 100 µm/mL Streptomycin for neuron differentiation, the differentiated cells were stained with β-tubulin III (Tuj1; BioLegend, San Diego, CA, USA) and Tropomyosin receptor kinase B (TrkB; Santa Cruz Biotechnology, Inc, Dallas, TX, USA). Nuclei were counterstained with 20 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma).

Colony-Forming Unit-Fibroblast (CFU-F) Assay

Culturing Time

After trypsinization with 0.05% Trypsin / EDTA, cells were resuspended in growth medium to disaggregate the clumps and produce a single cell suspension. Viability, cell number, and proportion of single cells were determined by Trypan Blue (Sigma). The cell suspension was seeded into 6-well culture plates at 250 cells per well. Cells were incubated for 12-14 days at 37°C in 5% CO_2 with 2 ml growth medium per well which was changed every 2 days.

Seeding Density

The SHED expanded in culture to 80-90% confluency were harvested with Trypsin/EDTA treatment and counted using a haemocytometer. A single cell suspension was diluted in growth medium, and they were plated into 6-well culture plates at various densities. After incubation at 37°C in 5% CO_2, the cells were washed with PBS and assayed for CFU-F.

FBS Supplement

Cells were seeded in triplicate into each well of a 6-well plate containing growth medium supplemented with 1% penicillin/streptomycin and 10% or 20% FBS Biochrom GmbH (Catalog no. S 0613/S 0615: FBS(Bio)) or Sigma St (Catalog no. F7524: FBS(Sig)). Cells were incubated at 37°C in 5% CO_2 with 2 ml growth medium per well which was changed every 2 days.

Ascorbic acid and L-ascorbic acid 2-phosphate supplement

A single cell suspension was seeded into a 6-well plate containing growth medium supplemented with 1% penicillin/streptomycin, the best concentration of FBS from the previous experiment and 0, 50, 100, 200 μM ascorbic acid (AA; Sigma St) or L-ascorbic acid 2-phosphate (A2P; Sigma St). Both AA and A2P were dissolved in SHED growth media. Cells were incubated at 37 °C in 5% CO_2 with 2 ml growth medium per well which was changed every 2 days.

Analysis

After 12-14 days, culture medium was removed and the cells were fixed for 20 min with 1% paraformaldehyde (Sigma St) in PBS buffer and stained with 0.1% toluidine blue O (Certistain Merck, Frankfurter, Germany) dissolved in 1% paraformaldehyde. The cells were washed with distilled water and colony morphology was inspected under a light microscope. The colonies larger than 2 mm in diameter were enumerated using ImageProPlus 7.0 program (Media Cybernetics, Rockville, MD, USA).

Statistical Analysis

All experiments in this study were performed 3 times. The data shown are representative results. Values were calculated as the means and standard deviations. The differences in colony numbers and sizes were analyzed by one-way ANOVA of variance using SPSS software (Version 10.0; SPSS, Chicago, IL, USA). The values at $p < 0.05$ were deemed significant.

Results

Isolation and Characterization of SHED

To isolate and identify MSCs in dental pulp tissue, the cells were extracted from the remnant pulp of exfoliated deciduous teeth after outgrowth method. The fibroblast-like cells began to migrate from pulp tissue into the flask by almost 7 days after seeding (Figure 1a) and reached 70-80% confluence by 20 days in growth medium with 10% FBS (Figure 1b). At passages 3-4, the quality of cells acquired was assessed using flow cytometry analysis for the expression of MSC surface markers. All cells were highly positive for the surface antigens CD44, CD73, and CD105 (Figure 1c). Moreover, a CD34 was not expressed on the cell population examined (Figure 1c). The differentiation potential of isolated cells is important considering their potential to regenerate specified tissues (Figure 1d). After 28 days of cultivation in osteogenic medium, the presence of calcium mineral was observed and confirmed by positive Alizarin red staining (Figure 1d). Moreover, the adipogenic differentiation capacity of cells was confirmed by the formation of lipid-filled vacuoles stained with Oil red O after 28 days of incubation in an adipogenic differentiation medium. To demonstrate chondrogenesis, the SHED were placed into chondrogenic medium for 21 days. SHED lines had the capacity to differentiate into chondrocytes (Figure 1d). The differentiated cells showed cartilage-specific proteoglycan and were positive for Alcian blue staining. Finally, fourteen days after transfer into neurons induction medium, the morphology of SHED changed from elongated to extension of the cell body into neuron-like processes and a spherical soma. The SHED expressed mature neuronal marker Tuj1 and all cell nuclei were positive for DAPI (Figure 1d).

Optimal Culture Time for Colony Forming Unit-Fibroblast (CFU-F) Assay

To determine the optimal time that is best suited for the formation of stem cell colonies, three SHED lines (SHED #1, #2, and #3) from exponential growth were plated at a density of 250 cells/well of 6-well plate and cultured for 12-14 days in DMEM supplemented with 10% FBS (Table 1). All counting colonies were larger than 2 mm in diameter. The average number of SHED colonies (from 3 wells of 6-well plate) on day 12 was 3 ± 0.33. The colony numbers were higher on day 13 (5 ± 0.77) and day 14 (17 ± 0.69) than on day 12 (Table 1). These data suggested that the optimal time point for SHED colony formation assay was 14 days.

Table 1. Number of SHED colonies in day 12-14

Day	The mean numbers of colonies (per 3 wells)			
	SHED #1	SHED #2	SHED #3	Mean ± SD
12	2.67	3.33	3.00	3.00 ± 0.33
13	4.33	5.67	5.67	5.22 ± 0.77
14	16.00	17.33	16.33	16.56 ± 0.69

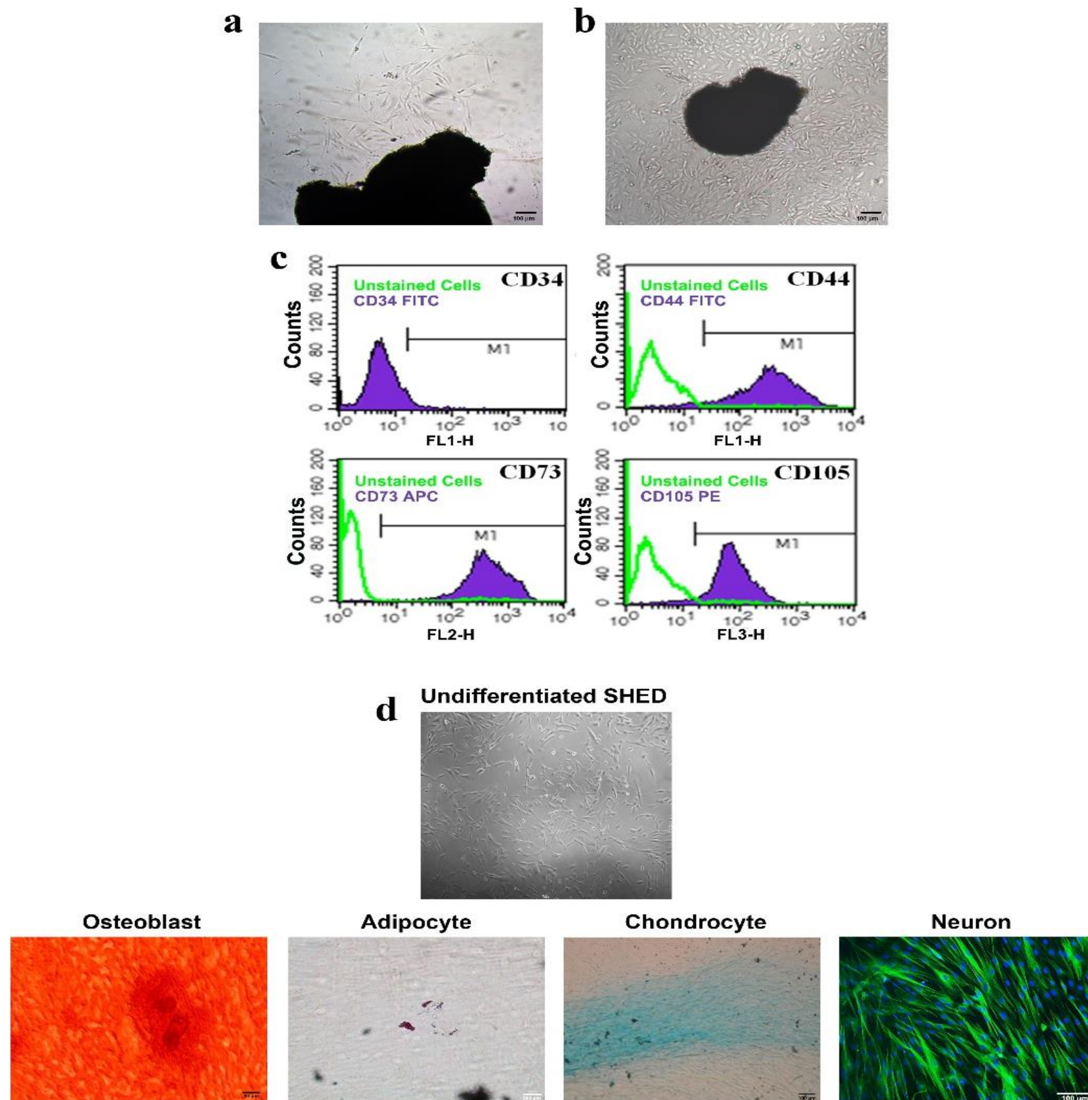

Figure 1. Isolation, morphological observation and characterization of stem cells from pulp tissue of human deciduous teeth. (a) Outgrowth of stem cells from dental pulp tissue of human exfoliated deciduous teeth. (b) Phase-contrast images showing the fibroblast-like morphology of the *in vitro* expanded SHEDs. (c) Flow cytometric analysis of the expression of mesenchymal stem cell markers CD44, CD73, CD105 and the hematopoietic marker CD34 on SHEDs. Gate M1 are deemed to be as positive. (d) Multi-differentiation of undifferentiated SHED, cells staining with Alizarin Red S for osteogenic differentiation, Oil Red O for adipogenic differentiation, Alcian blue for chondrogenic differentiation, and the mature neuronal marker Tuj1 (green) for neuronal differentiation. Cell nuclei were counterstained with DAPI (blue). Scale bars: 100 µm in a, b and d (chondrogenic and neuronal differentiation), 5 µm in d (undifferentiated SHED), 50 µm in d (Osteogenic differentiation), and 200 µm in d (adipogenic differentiation). SHED, stem cell from human exfoliated deciduous teeth, Tuj1, β-tubulin III, and DAPI, 4',6-diamidino-2-phenylindole.

Table 2. Seeding density and colony number of SHED

Seeding densities (Cells)	The mean numbers of colonies (per 3 wells)			
	SHED #1	SHED #2	SHED #3	Mean ± SD
100	4.33	4.00	4.33	4.22 ± 0.19
250	18.33	18.00	18.67	18.33 ± 0.33
500	28.00	25.67	25.33	26.33 ± 1.45
1000	ND	ND	ND	ND

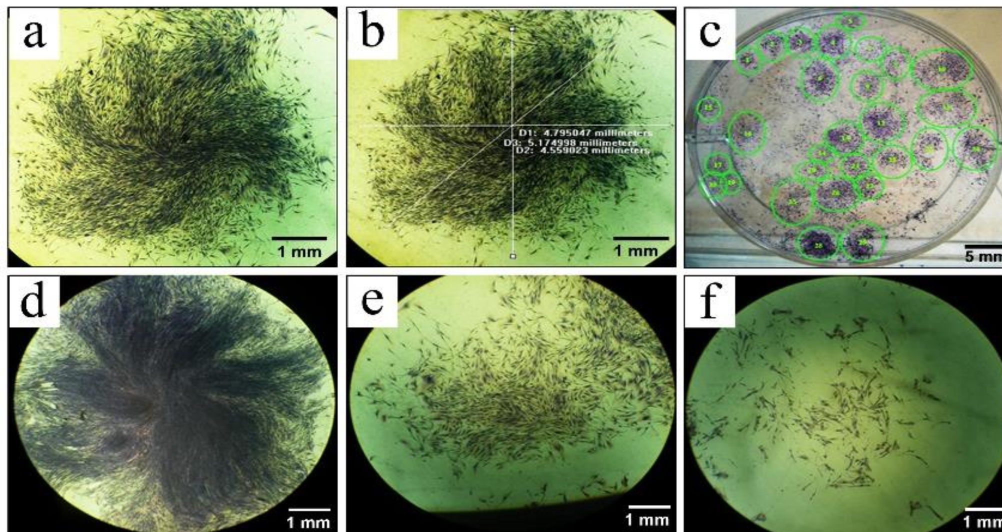

Figure 2. Morphology of SHED colonies stained by toluidine blue. (a) Phase-contrast images showing the morphology of SHED colony. (b) Assessing colony size using ImageProPlus 7.0 program. (c) Measurement of colony number by ImageProPlus 7.0 program which showing in green circle. SHED form colonies of three distinct morphologies; (d) stem cell, (e) transit-amplifying and (f) differentiated colonies. Scale bars: 1 mm in a, b, d, e, and f, and 5 mm in c.

Effect of Cell Seeding Density on the Formation of SHED Colonies

To determine the cell seeding density for optimal CFU-F assay, three lines of SHED were plated at densities of 100, 250, 500 and 1,000 cells/well of 6-well plates and cultured for 14 days in medium supplemented with 10% FBS (Table 2). All SHED lines attached to the plastic plates and had the capability to form fibroblasts-like colonies (Figure 2a). The easily distinguishable colonies greater than 2 mm in diameter were measured the colony size (Figure 2b) and the number of colonies (Figure 2c) using ImageProPlus 7.0 program. In normal growth medium, SHED cells formed three types of colonies. The holoclones or SC colonies were large consisting of many small, tightly packed cells that stained darkly (Figure 2d). The meroclones or TAC colonies were a mixture of cell types, small, tightly packed cells at the center and larger cells at the edges (Figure 2e). The paraclones or DC colonies were the smallest and consisted mainly of large, flattened and scatter cells (Figure 2f). In all densities, the TAC colonies were most abundant, followed by SC and DC colonies, respectively (Figure 2c). Increasing SHED seeding number (100, 250, and 500 cells/well) in 6-well plates resulted in a strong increase statistically significant in colony numbers (4 ± 0.19, 18 ± 0.33 and 26 ± 1.45 colonies, respectively) (Table 2). On day 14, the SHED seeding of 1,000 cells/well grew to a very high density and the colony number could not be counted (Table 2). These data suggested that the best cell seeding density for SHED colony assay was 500 cells/well of 6-well plate within 14 days.

Selection of optimal supplemented substrates for CFU-F assay

To define the optimal supplemented substrate for the colony-forming unit assay, the SHED seeding density at 250 and 500 cells/well of 6-well plate were further chosen since these densities provided the higher numbers of colonies. The SHED were cultured for 14 days in the presence of different medium supplements as described in Materials and Methods.

Fetal Bovine Serum

In this study, the FBS from 2 companies, Biochrom GmbH and Sigma St called FBS(Bio) and FBS(Sig), were selected for the evaluation of CFUs. Since all cell lines exhibited similar results, SHED #1 was used for further experiments. The experiments were repeated 6 times. The FBS concentration was first experimented for the effect on CFU-F (Fig 3). The TAC colonies were observed at the highest proportion of both 10% & 20% serum concentrations of Biochrom GmbH company (Fig 3a). In contrast, the colonies formed only DC colony type, which were found in the presence of 20% FBS(Sig) from Sigma St company (Fig 3a). The results were similar when seeding cells at a density of 250 & 500 cells/well in the presence of both FBS(Bio) and FBS(Sig) (Fig 3a).

When seeding cells at a density of 250 cells/well in the presence of 10% FBS(Bio), the number and size of CFU-F were lower compared to 20% FBS(Bio) with cell seeding at 500 cells/well (Fig 3b-c). The number of CFU-F-derived SHED were dependent on the concentration of FBS(Bio). For FBS from Biochrom GmbH, CFU-F in medium containing 20% serum had significantly higher number than 10% serum in both cell seeding densities. However, very few colonies (1-3 colonies) were observed at both the start of seeding SHED of 250 and 500 cells/well in the presence of FBS(Sig) (Fig 3b).

For colony diameter (Fig 3c), colonies in 20% FBS(Bio) were significantly larger than at 10% FBS(Bio) concentration when starting the cells density at 500 cells/well. While seeding density of 250 cells/well in the presence of FBS(Sig), the SHED colonies with average 2.60 mm in diameter was rarely found. The range of colonies size was increased with a diameter of 3.1-3.3 nm with cell seeding at 500 cells/well (Fig 3c). The largest number and size of CFU-F were obtained in 20% FBS(Bio) after cell seeding at 500 cells/well (Fig 3b-c). Therefore, we used only 20% FBS(Bio) from Biochrom GmbH company in subsequent experiments.

Ascorbic acid and L-ascorbic acid 2-phosphate

To observe the effect of ascorbic acid and it derivative on the forming of colonies, the SHED #1 was cultured in DMEM supplemented with 20% FBS(Bio) and 0, 50, 100, or 200 μM ascorbic acid or 0, 50, 100, or 200 μM L-ascorbic acid 2-phosphate for 14 days (Fig 4). The concentrations of ascorbic acid (vitamin C; AA) had large impact on the CFU-F of SHED (Fig 4). From the 250 cells/well seeding in culture medium supplemented with AA at 50 μM, there was a marked increase in the proportion of SC colonies (Fig 4a; upper row). However, the SHED seeding at 500 cells/well became over confluent and did not result in well-separated colonies (data not shown). All tested concentration of L-ascorbic acid 2-phosphate (A2P), a long-acting vitamin C derivative, also resulted in a reduction in the proportion of TAC colonies and an increase in the proportion of SC colonies. There was no DC colonies in medium supplemented with A2P (Fig 4a; lower row). Similar to the observation in ascorbic acid, there were no isolated colonies in SHED seeding densities of 500 cells/well (data not shown).

SHED in culture medium supplemented with AA at 50 μM, the number and size of colonies were increased compared to the medium with no AA (Fig 4b-c). The ascorbic acid at 100 and 200 μM had significant toxic effects toward SHED and very few colonies formed in both cell seeding densities (Fig 4a-b). On the other hand, the A2P had no toxic effect on SHED proliferation (Fig 4a, 4c). The A2P also did influence the colony size (Fig 4c) but small changes in colony number when using SHED seeding at 250 cells/well (Fig 4b). The sizes of colonies were gradually increased with increasing concentrations of A2P, with the biggest colonies observed at 100 μM (4.5-5.5 mm in diameter). The decline in a colony size was found in culture medium with 200 μM A2P (Fig 4c). The A2P was superior to AA as a supplement for SHED colony formation *in vitro* cultures (Fig 4). The data suggested that the highest CFU-F efficiency was obtained when SHED was cultured in growth media supplemented with 100 μM L-ascorbic acid-2-phosphate.

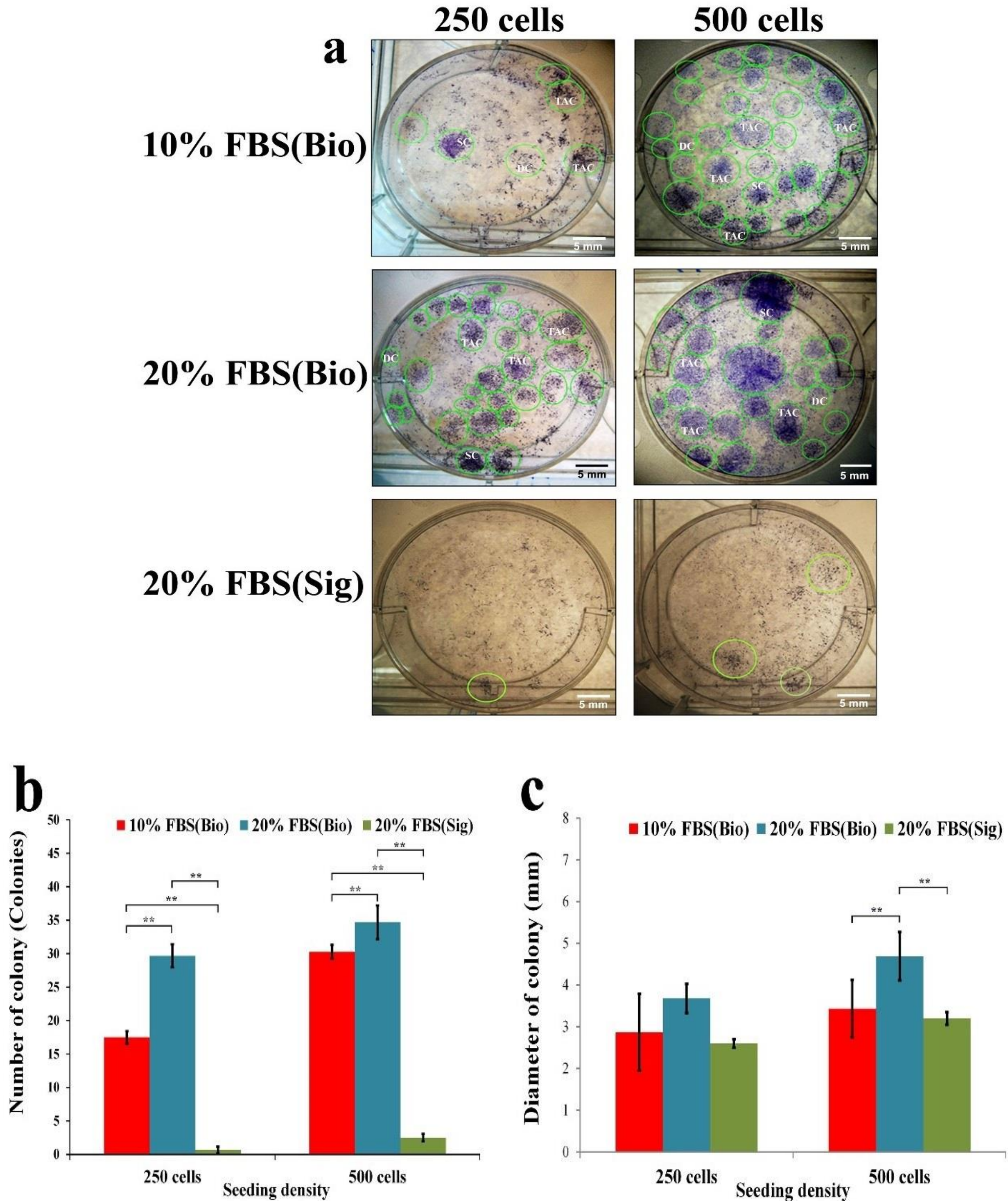

Figure 3. Influence of FBS on morphology, number, and size of SHED colonies. SHED at 250 cells and 500 cells per well of 6-well plated were cultured with 10% and 20% FBS from two companies (Biochrom GmbH; FBS(Bio) and Sigma St; FBS(Sig)). (a) Representative images of the colonies stained by toluidine blue after 14 days of cultured in 10% and 20% FBS from Biochrom GmbH company and 20% FBS from Sigma St company. Each colony in green circle. Scale bars: 5 mm. (b) The total number of colonies counted within each group. (c) The mean diameters per colony of SHED. The data are expressed as the mean ± SD of 6 replicate cultures (**p < 0.05). SC, stem cell; TAC, transit-amplifying; and DC, differentiated colonies; SHED, stem cell from human exfoliated deciduous teeth.

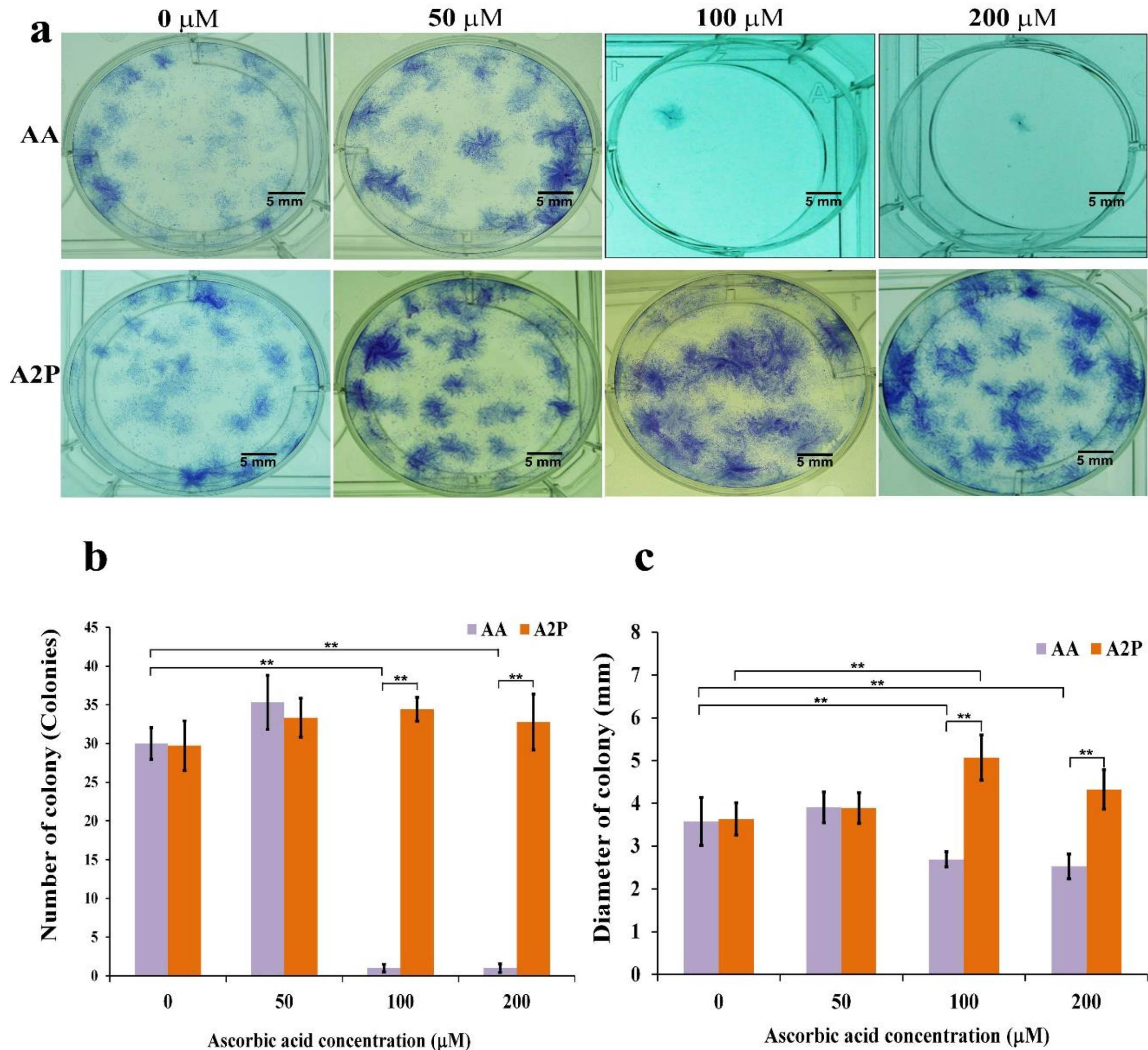

Figure 4. Influence of ascorbic acid and ascorbic acid 2-phosphate on morphology, number, and size of SHED colonies. SHED at 250 cells per well of 6-well plated were cultured in a range of concentrations between 0 and 200 μM. (a) Representative images of the colonies stained by toluidine blue after 14 days of cultured in 0-200 μM ascorbic acid (upper row) and 0-200 μM ascorbic acid 2-phosphate (lower row). Scale bars: 5 mm. (b) The total number of colonies counted within each group. (c) The mean diameters per colony of SHED. The data are expressed as the mean ± SD of 6 replicate cultures (**p < 0.05). AA, ascorbic acid; A2P, ascorbic acid 2-phosphate; SHED, stem cell from human exfoliated deciduous teeth.

Discussion

The SHED holds promise as unique resource for dental therapies including autologous stem-cell transplantation and tissue engineering and does not carry the ethical burden of embryonic stem cells. The CFU-F with the crucial character of stem cells enables it a long term self-renewal and rapid proliferation properties [3, 13] and an ability to differentiate [14]. The low initial number of CFU-F is a disadvantage. On the other hand, the accurate culture conditions of CFU-F are able to support proliferation and differentiation of stem cells for their future therapeutic uses. However, several factors were reported to have impact on the CFU-F assay including the time of culturing, cell-seeding density, and medium composition [15-17]. In this study, the highest CFU-F formation was obtained when SHED

was cultured at the density of 500 cells per well of a 6-well plate for 14 days in Dulbecco's modified Eagle medium (DMEM) with 20% FBS and 100 μM L-ascorbic acid-2-phosphate.

The influence of culturing time was first evaluated for the formation of CFU-F in SHED. Culturing time for CFU-F formation in stem cells from exfoliated deciduous teeth is still a controversial issue. It occurs between day 10 and day 14 [18-20], our work found that 14 days were adequate for the colony formation of SHED.

The CFU-F colonies exhibit heterogeneous proliferation and differentiation potentials, which are correlated with their heterogeneous morphologies [21-24]. Under normal growth culture condition, the SHED formed colonies of three morphological types: holoclones or stem cells (SC), meroclones or transit-amplifying colonies (TAC) and paraclones or differentiated colonies (DC) as previously described [6, 15]. The high number of cells in the SC colonies corresponds to the high proliferative potential and possess differentiation potential, whereas the DC colonies have a limited remaining proliferative capacity and are fully differentiated [6]. The TAC are in between these two colony types in terms of size and number of cells within the colony and have a mixture of characteristics. Under standard culture conditions, the colony morphology formed varies according to the cells line [15]. Here, the colony formed by SHED was shown to be TAC colonies with relatively high prevalence, followed by SC and DC colonies, respectively.

Previous reports about the critical parameters for human bone marrow MSC expansion have reported that the plating MSC density affects the proliferation and stemness preservation [5, 25, 26]. In addition, the starting population is crucial for the final numbers of cells to be obtained albeit the proliferative capacity of MSCs is very high [12]. Our results from the testing of four different plating densities, initial plating density of 250 cells and 500 cells/well of 6-well plate, gave much higher colony numbers of the starting SHED adherent population. Better yet, the SHED colony morphology was not critically altered when different cell densities are seeded as the highest colony's proportion was TAC.

The FBS plays a pivotal role in cell culture [16]. The concentration of serum affects the MSC propagation. Without the serum, the mouse bone marrow stem cells show senescence signs and died in a few days [27]. In 10% FBS media, mouse MSC proliferation is visibly increased and 20% FBS media has stronger effects on cell growth than 10% FBS media [16, 28]. This study showed that the number and size of SHED colonies in 20% FBS were significantly higher than those in 10% FBS. However, both FBS concentrations did not affect the morphology of colonies and the TAC colonies were still the most prevalence. The content of protein components such as bovine serum albumin and growth factors can vary widely between lots of the sera and possibly reduce or increase the CFU-F formation and cellular growth [29]. In our experiments, the FBS from two different companies (Biochrom GmbH and Sigma St) were tested and observed the colony formation after 14-day. FBS from Sigma St company significantly reduced the ability to promote the formation of SHED colonies and the morphology appeared to be more like DC colonies. Thus the commercial FBSs with variation in the concentrations and composition of components such as albumin, globulins, hemoglobin and total proteins can affect the proliferation and morphology of SHED colonies. Inconsistent cell growth can be due to the different lots of FBSs used which has varying concentrations of various proteins particularly the growth stimulatory factors, growth inhibitory factors and other growth related proteins [30].

In addition, we chose to study the ascorbic acid (AA) and ascorbic acid 2-phosphate (A2P) which are essential supplements in a variety of cultures [31-34]. One of the essential functions of AA is its role as a cofactor for the hydroxylation of proline and lysine residues in collagen, which is the most abundant protein in the body [35]. The *in vitro* proliferation and differentiation of MSCs into skeletal tissues requires ascorbic acid as an essential medium component [34]. In some cell culture conditions, the ascorbic acid concentrations of 0.01–0.5 mM inhibit or stimulate the proliferation of various kinds of cells including HEP2, KB, ascites, bone marrow and melanoma cells [34, 36-38]. The AA can induce morphological changes and apoptosis in a cell culture of chondrocytes after 18 h of cultivation [39]. Moreover, the numbers of leukemic cell colonies grown in

culture are reduced by the addition of L-ascorbic acid (0.3 mM) to the culture medium [40]. When 50 µM AA was added in the cultivation of SHED, it stimulated the SHED colony formation and growth and increased in the proportion of SC colonies. On the contrary, the AA at 100 and 200 µM repressed nearly 100% of SHED colony formation. This inhibitory effect may be due to several possibility. There may be the accumulation of H_20_2 and the formation of lipid peroxide in mitochondria that lead to cellular damages [5, 40-43]. The AA may induce an increasing level of cyclic adenosine 3':5'-monophosphate that inhibits cell growth [44]. Moreover, the AA is unstable in solution, especially under the normal culture conditions of neutral pH and 37°C. Instead of AA, an ascorbic acid 2-phosphate (A2P), a phosphate derivative of AA, has been used as a supplement in a variety of cultures [31, 45, 46]. In human fibroblasts culture, the A2P has a cofactor activity for collagen biosynthesis [47]. Moreover, it is very stable under the culture conditions [47]. Similar to osteoblast cells [33], the A2P up to 200 µM has no inhibitory effect on SHED growth. The size of SHED colonies and the proportion of SC colonies were increased by the A2P. The A2P is also resistant to oxidation by oxygen. It releases free AA by the action of alkaline phosphatase in the cell membranes of most animal cells. Our data suggested that the A2P-supplement was an effective way to help increase the formation of undifferentiated SHED colonies.

Collectively, this study provides the general guidelines for the establishment of a standardized protocol aiming at the preparation of high-quality adherent dental stem cells from human exfoliated deciduous teeth clusters (or colony forming units-fibroblastic) for use as source for preclinical and clinical studies.

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Ethical Compliance

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This study was conducted in accordance with the ethical committee of the Faculty of Dentistry, Mahidol University (IRB 2014/044.2710).

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