

Research Article

Stem cells from apical papilla and their properties in two primary culture methods

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Abstract

Background and Objective: Stem cells from the apical papilla (SCAP) are a unique population of dental mesenchymal stem cells and also different to dental pulp stem cells. Study and application of this cell type are extremely important in natural tooth root regeneration, especially in the treatment of oral diseases. Studies have isolated and characterized stem cell properties of SCAP by only one culture method- enzymatic dispersal method. The purpose of this study was to compare two primary culture methods in isolation and characterization of SCAP.

Materials and Methods: The effects of two methods on the cell viability, colony-forming efficiency, proliferation rate and multilineage differentiation potential and profiles of mesenchymal stem cell markers *in vitro* have been examined.

Result: We found that cultured cells adhered to the surface and developed with the same proliferation rate and formed colony units in both methods. In the enzymatic dispersal method, time for primary culture is shorter; differentiation potential is also higher than in outgrowth method. However, the cultured population of cells by outgrowth method is more homogenous with high expression of CD44, CD73, CD90 and negative with CD34, CD45, HLA-DR (<2%).

Conclusion: We conclude that two methods are available for primary culture of SCAP and in different situations; we should use more appropriate method.

Keywords: SCAP, outgrowth method, enzymatic dispersal method

1. Introduction

Dental stem cells are mesenchymal stem cells, which play an important role in tooth development, especially in clinical treatments for dental diseases. Stem cells from the apical papilla (SCAP) are found in the developing tissue- apical papilla of immature permanent teeth. Study of dental stem cells helps to regenerate full tooth like its development in nature. Dental pulp stem cells (DPSC) have potential roles in dental pulp regeneration, periodontal ligament stem cells (PDLSC) are able to regenerate periodontal ligament tissue, and SCAP are potential members for recovery of tooth root.

Stem cells from apical papilla that are population of multipotent stem cells have been isolated from the human apical papilla. According to studies in laboratories on animals and clinical observations, two populations of mesenchymal stem cells derived from the apical papilla of human immature permanent teeth-SCAP and from human dental pulp stem cells - DPSC have an important role with great potential in wound healing of incompletely developed teeth. In addition, the expansion apical apex in immature teeth is favorable factors help circulation in heart development in the marrow cavity, carrying the stem cell and tissue regeneration to facilitate ivory marrow in the marrow cavity. SCAP have telomerase expression at high level. This protein plays an important role in the activity of stem cell division. Both SCAP and DPSC have the potential to differentiate into bone, dentin as the mesenchymal stem cells derived from bone marrow. Soft tissue from the outer apical papilla have shown positive for markers STRO-1 and CD24-surface markers of SCAP, this marker will be lost during cell differentiation and generate dentin. Compared with DPSCs, SCAP have a number of cells which is positive for STRO-1 with higher level, have faster cell division, their population doubling level and the ability to create dentin *in vivo* is higher. Furthermore, studies have shown that population of stem cells derived from this tissue is positive with characteristic markers of mesenchymal stem cells, such as CD44, CD73, CD90, CD105, CD106, and CD146 ... and negative with markers of hematopoietic stem cells, mature blood cells such as CD18, CD34, CD45, HLA-DR⁷. Compared to DPSCs and other types of mesenchymal stem cells, SCAP are positive in telomerase activity- a sign of embryonic stem cells and they may be the source of very young cells for applications in hard tissue regeneration, this has been demonstrated through a number of experiments about creating bio-root in minipig. Many researches about the isolation and characterization of SCAP had performed, they demonstrated stemness of cultured cells derived from apical papilla such as proliferation, differentiation potentials, expression of MSC markers and many experiments investigated the application of SCAP. However, in most of these studies, cells were collected from the enzymatic dispersal culture method. We conducted this study to check whether or not different properties in two primary culture methods: enzymatic dispersal method and outgrowth method. We examined cells properties base on features: proliferation, CFU-F forming efficiency, MSC marker expression, and differentiation potential.

2. Materials and methods

2.1 Sample collection

Healthy human molars with immature roots were harvested from patients at the Maxillo-facial Faculty, under the approval of the Ethical Committee of Ho Chi Minh City Medicine and Pharmacy University. After extraction, the teeth were immediately soaked in DMEM (Sigma) supplemented 300 IU/ml penicillin; 300 µg/ml streptomycin; 0.75 µg/ml amphotericin B (fungizone®). The apical papilla tissue must be isolated and cultured within 24 hours.

2.2 Cell culture

SCAP cultures were established by two distinct approaches: outgrowth method and enzymatic dispersal method.

2.2.1 Outgrowth method

The apical papilla was gently separated from the surface of the root. The tissue was then washed several times in 1X PBS (Gibco) supplemented antifungal antibiotics on the level of dilution 4X, 3X, 2X, 1X and minced with scissors into small pieces (2-3 mm²). Each piece of tissue was transferred to 35 mm dish before a lamelle is laid over.

2.2.2 Enzymatic dispersal method

The apical papilla was minced finely with scissors and then treated with a solution of 3 mg/mL type I collagenase (Sigma) and 4 mg/mL dispase (Sigma) for 45 min at 37°C. After enzymatic digestion, single cell suspensions of SCAP were obtained by passing through a 70 µm cell strainer (BD Falcon). The cell suspension was then centrifuged at 3000 rpm for 5 minutes and seeded at 1×10^5 into 35 mm dish.

For both methods, the culture medium was DMEM/F12 (Sigma) supplemented with 10% FBS and 2 mM L-glutamin, 100 UI/ml penicillin, 100 µg/ml streptomycin. Cells were maintained in 5% CO₂ at 37°C. The medium was changed after 2 or 3 days. Cells were sub cultured when they reached 70 - 80% confluence. Cells used for all experiment in this study were at fourth passage.

2.3 Cell proliferation

Growth curves were performed to compare the proliferative potential of cells obtained by the outgrowth method and the enzymatic dispersal method. After four culture passages, cells obtained from both methods were plated at a density of 7×10^3 cells per well in 96-well plates with DMEM/F12 supplemented with 10% FBS and 2 mM L-glutamin, 100 UI/ml penicillin, 100 µg/ml streptomycin. Cell growth was assessed by Neubauer counting chamber method. Every 24 hours, the cells were harvested by trypsin-EDTA treatment and then were counted with a hemocytometer. The viability of cells was determined by the trypan blue dye exclusion test. For both isolation methods, these assays were repeated 3 times each day. The determination was carried out for 10 days.

The time of duplication or doubling time was assessed by the following equation: $T = N \times \ln 2 / \ln(A/a)$, where T = The time of duplication (hour), N = The time of log phase, A = final number of cells, a = initial number of cells.

2.4 Colony formation

For the colony-forming efficiency assay, cells were seeded into 35 mm dishes at low densities of $2-2.5 \times 10^2$ cells per dish. Cells were maintained in 5% CO₂ at 37°C until colonies were observed under microscope (aggregates of ≥ 50 cells were scored as colonies). The cell clones were then fixed with 100% ethanol for 10 minutes, and stained with 1 ml crystal violet for 30 minutes. Finally, the cells were washed with distilled water and the number of colonies was counted under inverted microscope. Experiments were carried out 3 times for each isolation method.

2.5 Flow Cytometry

After 4 culture passages, when cells reached 80 - 90% confluence, FACS analysis was carried out to characterize the SCAP population *in vitro* by specific surface markers. Cells were released using 0.25% trypsin/EDTA, then 10^6 cells were aliquoted, resuspended in 1 ml FACS Flow and incubated with specific monoclonal antibodies against CD34, CD45, HLA-DR, CD44, CD73, CD90 for 30 minutes at room temperature. Finally, the cells were analyzed by fluorescein-activated cell sorter (FACS) Calibur flow cytometry with Cell Quest Pro software.

2.6 In vitro differentiation of SCAP

To determine the multilineage differentiation potential of SCAP, fourth passage cells were seeded in 35 mm dishes and cultured in DMEM/F12 (Sigma). When the cells reached 70 - 80% confluence, the basic medium was replaced with a specific differentiation medium in order to induce differentiation capacity of SCAP. Cells cultured in DMEM/F12 was used as a control group.

For osteogenic differentiation, the cells were incubated in a medium consisting of DMEM/F12 supplemented with dexamethasone, L- ascorbic acid 2-phosphate and β -glycerol phosphate. After 21 days of culture, osteogenic differentiation was analyzed via alkaline phosphatase activity assay by staining with Alizarin Red S.

For adipogenic differentiation, the cells were cultured in an adipogenic medium containing DMEM/F12 supplemented with dexamethasone, IBMX, indomethacin and insulin. The presence of intracellular lipid droplets were observed during 21 days of culture under microscope with 200x or 400x magnification and confirmed by Oil Red O staining to assess adipogenic differentiation.

2.7 Statistical analysis

Student's t-test was used to analyze the differences between groups. P values less than 0.05 were considered as statistically significant.

3. Results

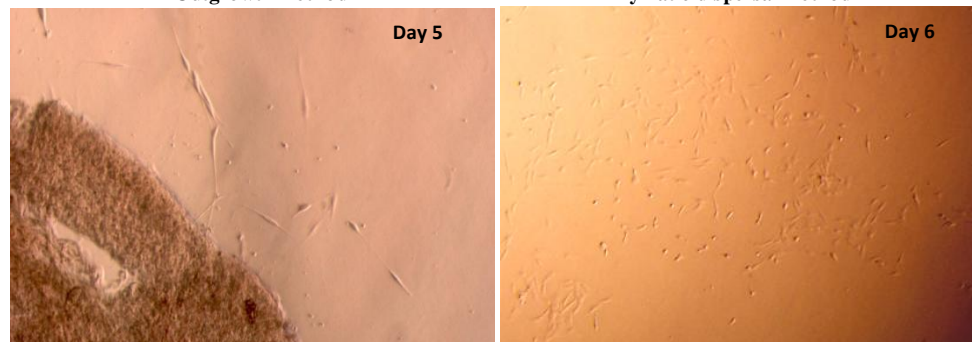
3.1 Primary culture of SCAP

SCAP were obtained by two different methods: outgrowth method and enzymatic dispersal method. For both isolation approaches, sample contamination with microorganisms was not observed and the majority of cells growing presented fibroblast-like morphology seen under inverted microscope (Fig.1). In the outgrowth methods, cells started to migrate from the apical papilla after 5 days of culture. Cells closer to the fragment were heterogeneous at the morphological level. After two weeks of culture, most cells appeared aligned, presenting long extensions of cytoplasm and organizing monolayers. Cells reached over 90% confluence at the 24th day. For the enzymatic dispersal, one-to-two days after plating, the cells started to adhere to a plastic dish and reach over 90% confluence after 20 days of culture, faster than cells obtained with the outgrowth. However, there are no differences in the morphology of adherent cells were observed between the two methods.

3.2 Cell proliferation

Considering the proliferation of SCAP, growth curves (Fig. 2) showed that SCAP obtainment with the two methods were all effective but the cell number was statistically higher when cells were isolated by the outgrowth method. For both methods, the cell number was highest after 4 days of culture. The calculated doubling times were 50 hours for the outgrowth and 51 hours for the enzymatic dispersal.

Figure 1. Primary culture of SCAP comparing outgrowth method and enzymatic dispersal method. Magnification: 40x.



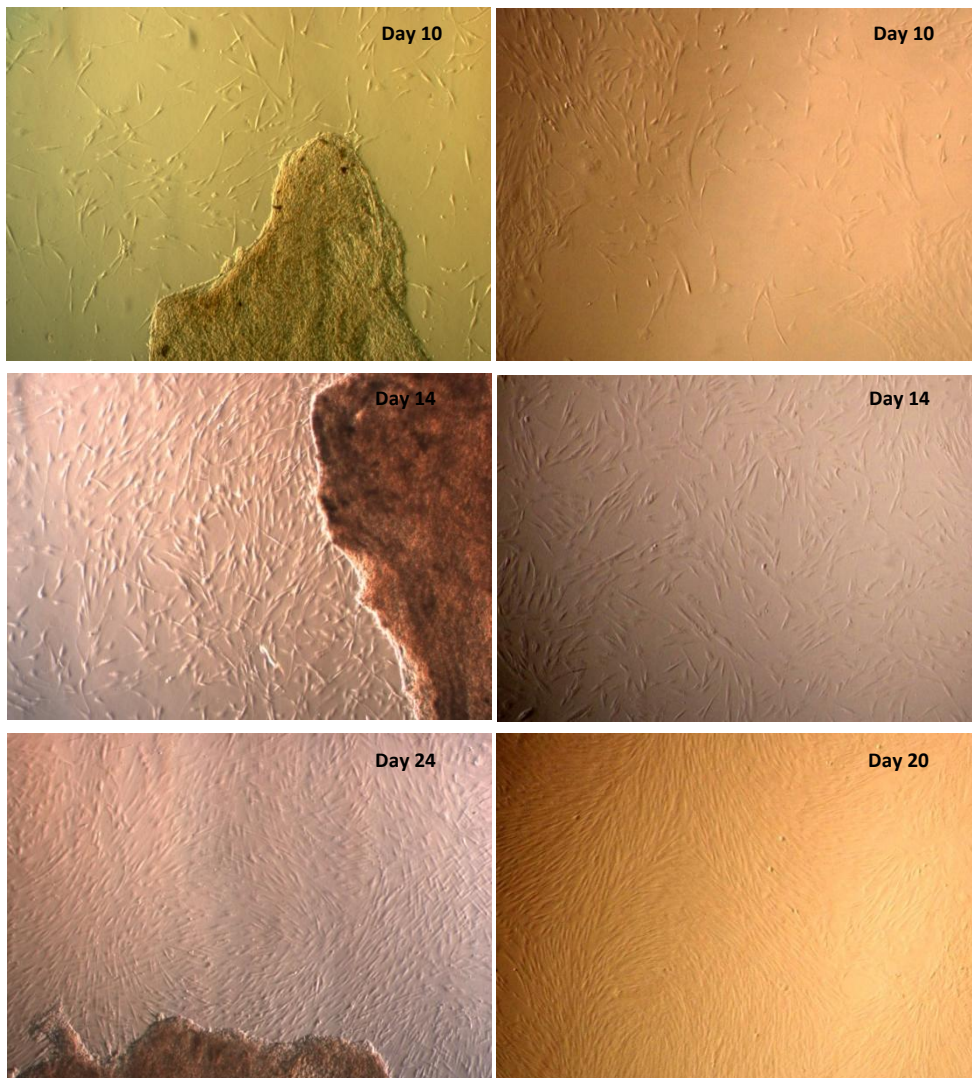
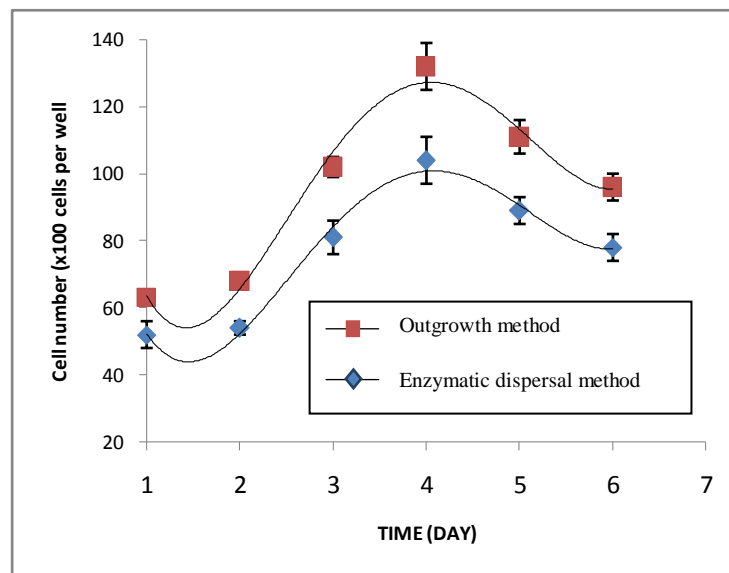


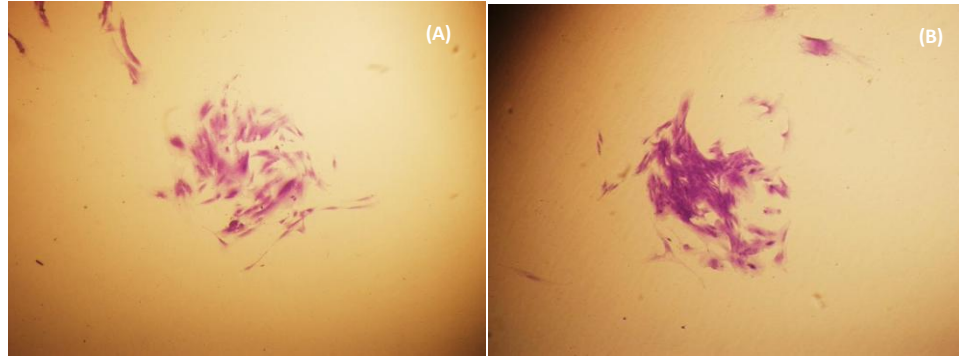
Figure 2: SCAP growth curves comparing outgrowth method and enzymatic dispersal method.



3.3 Colony formation

Colony-forming assay were carried out for cells obtained by both isolation method, the outgrowth and the enzymatic dispersal. The results demonstrated the presence of colonies of SCAP with typical fibroblast-like morphology of the cells in both group examined with no significant difference in colony-forming efficiency. Colonies of cells obtained by the outgrowth were observed after 10 days of culture and 12 days for cells obtained by the enzymatic dispersal.

Figure 3: Colony formation assay. (A) Single colony of SCAP obtained by outgrowth method after 10 days in culture; (B) Single colony of SCAP obtained by enzymatic dispersal method after 12 days in culture. Magnification: 40x.



3.4 Flow Cytometry

To characterize SCAP population *in vitro*, the cells were harvested and incubated with specific monoclonal antibodies against CD34, CD45, HLA-DR, CD44, CD73, and CD90. Flow cytometry analysis confirmed that cells obtained by both isolation methods were strongly positive (more than 95% positive cells) for the mesenchymal stem cells surface markers CD44, CD73, CD90 (Fig. 4). Cell-surface markers that were negative were related to hematopoietic stem cells surface markers CD34; mature blood cells and white blood cells surface markers CD45; lymphocyte, monocyte and macrophages surface markers HLA-DR. For cells obtained by the outgrowth, the analyses revealed a negative expression of these markers with the results of less than 2% positive cells. However, for the enzymatic dispersal, the percent of positive cell was higher (10% - 30% positive cells) (Fig. 4).

Figure 4: Flow cytometry analysis of the expression of cell surface markers of SCAP obtained by (A) the outgrowth method and (B) the enzymatic dispersal method.

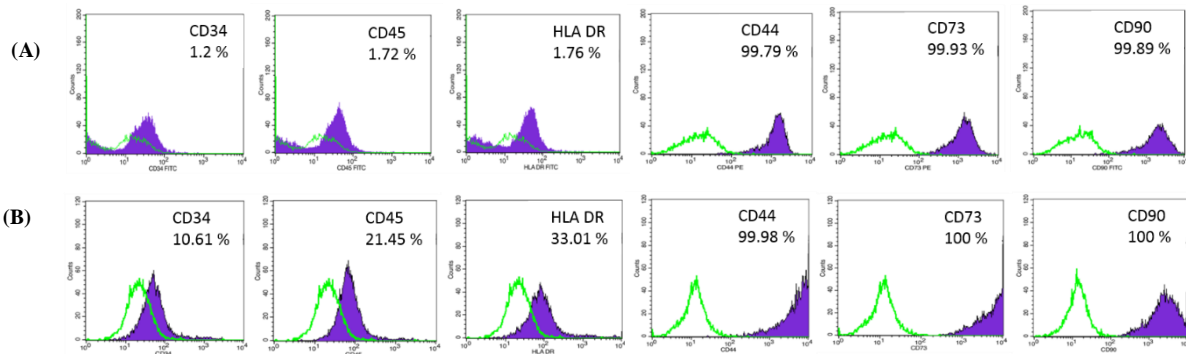
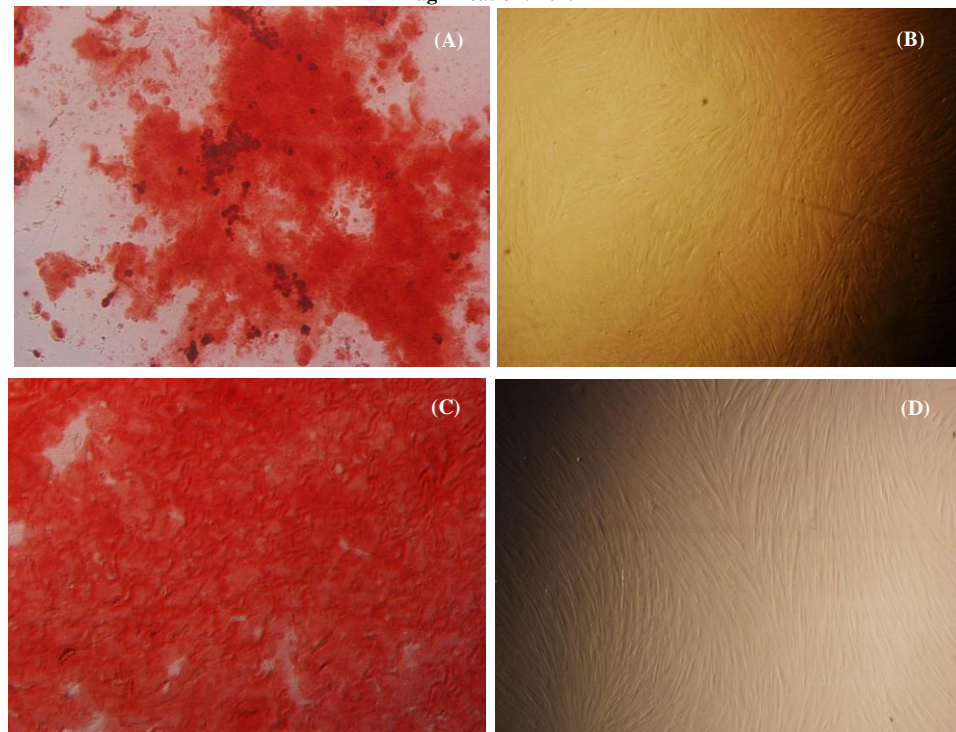


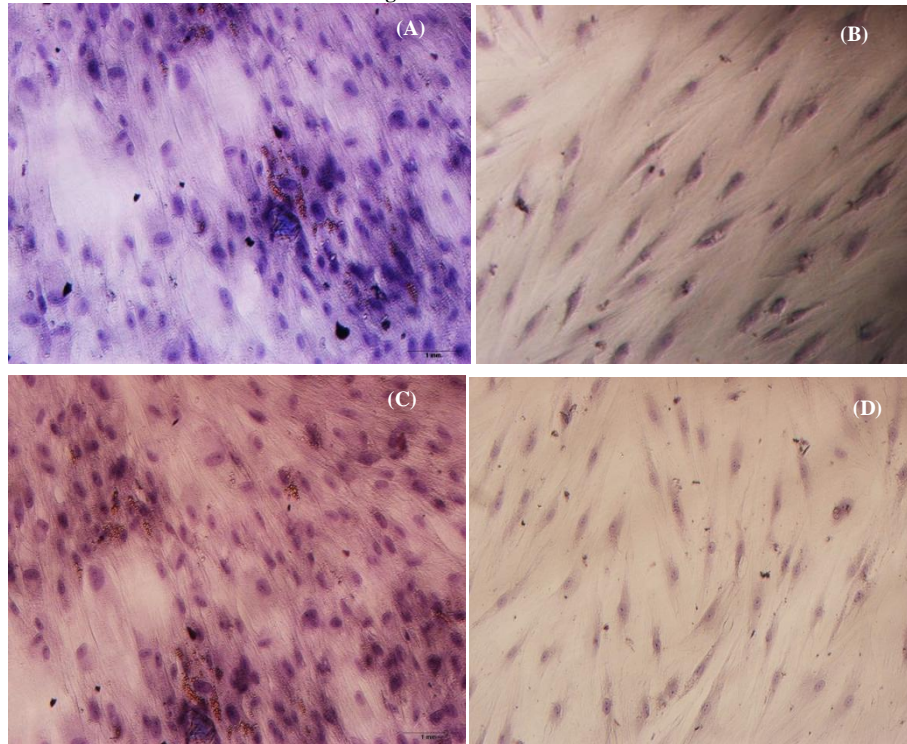
Figure 5: Odontogenic differentiation of SCAP after 21 days of culture. (A) The outgrowth; (C) The enzymatic dispersal; (B), (D) Control group. Magnification: 40x.



3.5 *In vitro* differentiation of SCAP

To determine the capacity of SCAP to undergo osteogenic and adipogenic differentiation, cells were cultured in specific differentiation medium. SCAP odontogenic differentiation was characterized by the formation of mineralized nodules after 21 days of culture that stained red with Alizarin Red S, compared with the controls, indicating calcium accumulation *in vitro* (Fig. 5). Adipogenic differentiation could also be obtained from SCAP cultures showed by the potential to develop into Oil Red O – positive lipid-laden fat cells after 14 days of culture for the enzymatic dispersal and 21 days for the outgrowth (Fig. 6).

Figure 6. Adipogenic differentiation of SCAP.(A) The outgrowth on 21st day; (C) The enzymatic dispersal on 14th day; (B), (D) Control group. Magnification: 200x.



4. Discussion

In our study, we demonstrated that cells from apical papilla tissue adhered, proliferated, and have a number of mesenchymal stem cell properties, including CFU-F forming capacity, osteogenic and adipogenic potentials and expression of CD44, CD73, CD90, that results is similar to the study of Sonoyama *et al* (2006). In the first step- primary culture, both methods showed that cultured cells have the ability to proliferate well. CFU-F forming assay results showed that cell populations obtained by both methods are capable of adhering proliferation, creating a cluster of cells from a single cell after one week. Time for cells cover entire culture dish in enzymatic dispersal method (15-20 days) is faster than in the outgrowth method (24-28days). After 3-4 times of subculture, cells have identical shape similar to the shape of mesenchymal stem cells obtained from apical papilla tissue in a study published by Sonoyama *et al* (2008). However, population doubling time of the cells isolated by the enzymatic dispersal method was 51 hours, while in outgrowth method is 50 hours. Investigation results showed that cells proliferated similarly in different culture methods with the same initial density. Cells obtained by outgrowth method expressed negatively (<2%) with markers often appear on hematopoietic stem cells-CD34; mature blood cells, white blood cells-CD45; lymphocytes, monocytes, macrophages- HLA-DR, these markers are not expressed on the surface of mesenchymal stem cells. However, this rate of these markers was higher in cell populations obtained by enzymatic method. In enzymatic method, apical papilla tissue with inherent vascular components are separated into single cells by enzymes and cells derived from blood vessels may adhere easily to surface of the culture dish, remaining after subcultures.

In outgrowth method, apical papilla is structural integrity with blood vessels in this tissue, only cells with stronger vitality moved from the edge of the tissue and adhered to the surface, so the contamination of cells derived from blood was lower. The results of the surface marker analysis showed that outgrowth method gained more purified cell populations compared with the enzymatic method. After 14-21 days, cells in the fourth passage developed into adipocytes and bone cells when cultured in different induction mediums respectively. After 5 days of adipogenic induction, cells isolated by enzymatic methods have an accumulation of small fat droplets in cells, the shape began to change, not long and fusiform shape as fibroblasts, cells became round and nuclear was pushed to one side; while fat droplets in outgrowth method was 10 days. After 14 days, fat drops in enzymatic method were bigger, we used Oil Red O Stain to identify adipocytes and 21 days with cells from the outgrowth method. The results showed that cells in outgrowth method have the lower differentiation potential than cell line cultured by enzymatic methods. Alizarin Red S staining was performed in two cell lines from two methods after 21 days of induction. However, cell line isolated by enzymatic methods have a higher level of differentiation, the dye stained on the entire surface of the dish, and only a part of cells were mineralized and stained with Alizarin Red S in outgrowth method. Through the above results, the population of cells derived from human apical papilla tissue is capable to differentiate into bone and fat cells under appropriate induction conditions similar to the potential of mesenchymal stem cells. This result is available to studies published by Sonoyama *et al* (2008). Both methods have different advantages and disadvantages. The outgrowth method obtained purified cell population, but took more time and less differentiation potential than cell lines in enzymatic methods. Thus, depending on conditions and demand of experiments, we can use more appropriate method to achieve optimal results, especially in basic researches and clinical treatments in oral diseases.

5. Conclusion

Our study has successfully isolated and cultured cells from apical papilla tissue in two methods: outgrowth method and enzymatic dispersal method. We also demonstrated the proliferation, the presence markers of human mesenchymal stem cells and differentiation potential of cultured cells from this tissue. We concluded that there is a population of mesenchymal stem cell in cultured cells.

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