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The Effect of Cell Source on Mesenchymal Stem Cell Behavior and Osteogenic Differentiation of Buccal Fat Pad and Dental Pulp Stem Cells, an *In Vitro* Study

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Abstract:

Objective: The buccal fat pad is a source of adipose stem cells accessible from the oral cavity and dental pulp is a common dental source of stem cells. This study aimed to assess the biological behaviors and osteogenic differentiation of human buccal fat pad adipose-derived stem cells (hBFP-ADSCs) and human dental pulp derived stem cells (hDPSCs).

Material and Methods: Buccal fat pads (595.4±80 mg) and dental pulp from 2 wisdom teeth were obtained from 3 matched donors and hBFP-ADSCs and hDPSC cells, respectively, and isolated. The isolated cells were sequentially expanded through passages 5, 10, and 15 for analysis. Cell growth, osteogenic differentiation, and cell senescence were investigated.

Results: HBFP-ADSCs and hDPSCs exhibited different cell morphologies and behaviors. Cell expansion was associated with the decrease of cell growth and *in vitro* mineralization of hDPSCs. The cell yields of hBFP-ADSCs and hDPSCs at the primary passage were $3.2 \times 10^5 \pm 9.9 \times 10^3$ cells/100 mg and $1.13 \times 10^6 \pm 2.4 \times 10^5$ cells/tooth, respectively. The expanded cells exhibited a limited life span and maintained normal karyotypes at the late cell expansion stage.

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Conclusion: HBFP-ADSCs showed steady cell growth, high osteogenic differentiation potential, tissue availability, and minimal *in vitro* cell expansion. The availability and accessibility of hBFP-ADSCs would enable clinical applicability as a stem cell source for bone regeneration.

Keywords: cell growth kinetics, human buccal fat pad, human dental pulp stem cells, mesenchymal stem cells, osteoblastic differentiation

Introduction

Cell-based bone tissue engineering has been extensively studied as an alternative treatment to autogenous bone grafting for bone regeneration in recent decades.[1] The transplanted mesenchymal stem cells may function as bone-forming cells or secrete trophic factors to promote osteogenesis.[2] Stem cell transplantation decreases donor site morbidity and patient discomfort as compared with autogenous bone graft harvesting.[3,4] Key factors contributing to the clinical applicability of stem cell sources include a large number of cells for stem cell transplantation[5-7], the availability, accessibility, and feasibility of using mesenchymal stem cells, ease of isolation and characterization, the possibility of directing cell differentiation into the target cells, and cell behaviors after cell expansion.

Human mesenchymal stem cells are capable of forming bone and promoting osteogenesis, and the common source in the oral cavity are dental tissue[7,8], and adipose tissue from the buccal fat pads.[9,10]

The buccal fat pads are encapsulated in the cheeks and can be easily accessed through the oral cavity for a common and convenient source of adipose tissue.[10,11] The characteristics and bone regeneration capacity of human buccal fat pad adipose-derived stem cells (hBFP-ADSCs) are similar to human subcutaneous adipose tissue-derived stem cells (SC-ADSCs).[9,12] Human ADSCs have been transplanted in patients with ß-tricalcium phosphate particles for craniofacial bone defects with and without bone morphogenetic protein-2 (BMP-2)[5,13], with organic bovine bone mineral for vertical and horizontal augmentation of atrophic posterior mandibles[14], and with iliac bone grafts covered with human amniotic membranes loaded with hBFP-ADSCs maxillomandibular bone defects[15] and demonstrated good outcomes.

The availability of hADSCs from accessible sources such as buccal fat pads makes them more versatile than tissues from a dental source. The advantages of hBFP-ADSCs over human dental tissue-derived stem cells are that buccal fat pads can provide a larger amount of tissue for stem cell isolation than dental tissues, and they are not restricted to extracted teeth.[10,12,16]

Human dental pulp stem cells (hDPSCs) are a well-established source of mesenchymal stem cells which can be isolated from the pulpal tissue of permanent teeth, in particular the third molars.[8,17] They provide multipotent mesenchymal stem cells [8,16] that are highly proliferative and able to form bone in the subcutaneous tissue of rats and human tooth sockets.[7,18] Although their availability is superior to other dental sources such as periodontal ligaments, dental follicles, and apical papillae, their versatility is limited and requires extensive *in vitro* cell expansion to generate a sufficient quantity of mesenchymal stem cells for autologous cell transplantation.[16,19] Moreover, extensive *in vitro* cell expansion can decrease cell growth and differentiation potential, increase cell senescence or result in a state of permanent cell-cycle arrest[20,21], and lead to spontaneous mutation of the expanded cells.[22] Limited tissue availability is another limiting factor for the clinical applicability of hDPSCs. The behaviors and characteristics of stem cells such as cellular doubling time (the time required for a cell population

to double), cell senescence (a state of permanent cell-cycle arrest), and cell proliferation and differentiation are influenced by the anatomical origin and levels of cell expansion.[23, 24] Buccal fat pad harvesting is safe and not complicated and can be done under local anesthesia. A previous animal study reported that ADSCs had a higher growth rate and better senescence resistance in culture, but the regenerative capacity to grow new teeth in the extraction socket was similar to hDPSCs.[25] The present study aimed to assess the potential of hBFP-ADSCs and hDPSCs in directing cell differentiation into bone tissue, cell behaviors after cell expansion in terms of cell growth, osteogenic differentiation potential, and stem cell expandability.

Material and Methods

Isolation and cell culture

Under the review and approval of the Ethics Committee of the Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand (EC5609-22-P-LR), with written patient informed consent, buccal fat pads and pulpal tissue were harvested from three healthy donors, aged 17-25 years old, who underwent surgical removal of the upper and lower impacted third molars. The patients were not taking any medications. After complete removal of the impacted upper third molars, the distal portion of the buccal fat pad was harvested through a vestibular incision distal to the third molar.[10] Dental pulp tissue was obtained from the upper and lower third molars of the same patients (Figure 1).

In brief, the hBFP and dental pulp tissue were washed in normal saline, minced, and digested in culture medium with enzyme solutions, with the hBFP-digested in 0.1% type I collagenase[26] and the dental pulp in 3 mg/ml collagenase type I and 4 mg/ml dispase (all from Roche Diagnostics, Mannheim, Germany).[8] The incubation was performed under gentle agitation in a humidified incubator at 37 °C under 5.0% CO_2 for 1 hr. Then 2 ml of growth medium containing 10.0% fetal bovine serum (FBS) was added to each sample to stop the reactions and the mixtures centrifuged at 300 g (Refrigerated Centrifuge, Labofuge 400 R, Kendro Laboratory Products, Osterode, Germany) for 10 min at 4 °C to obtain cell pellets. After that, each pellet was dispersed in 10 ml of growth medium and seeded in T75 flasks, approximately 150 mg of the buccal fat pad and pulpal tissue from one tooth for one T75 flask, and cultured in a humidified incubator at 37 °C and 5.0% CO_2. The medium was changed on day 3 and every 2-3 days thereafter. On day 10-13 after cell seeding, when the expanded cells reached 80.0% confluence, the cells were passaged and seeded at $3x10^3$ cells/cm^2 in a growth medium for cell expansion. At passages 5, 10, and 15, the cells were cultured in a growth medium comprised of α-MEM, 10.0% FBS, 1.0% penicillin, and 0.1% fungizone (all from Gibco, Thermo Fischer Scientific, Waltham, MA, USA) for growth kinetics, cell senescence, cytogenetic analyses, and osteogenic medium for osteogenic differentiation studies.

The osteogenic medium was a growth medium supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma-Aldrich, St. Louis, MO, USA).[27,28]

Cell proliferation and doubling time

Human BFP-ADSCs and DPSCs were seeded on 6-well cell culture plates at $2x10^3$ cells/cm^2 in 2 ml of growth medium. On days 1, 3, 5, 7, and 9 after cell seeding, cells were trypsinized and counted using hemocytometers (BLAUBRAND® Neubauer Pattern Counting Chamber, Sigma-Aldrich). Then growth curves were constructed and doubling time (DT) was calculated as (ln [N/N0])/(ln 2), where N represents the number of counted cells and N0 represents the number of plated cells. The number of samples was 4 samples per group at each investigation time (n=4).[29]

Cell senescence

Human BFP-ADSCs and DPSCs were seeded on 6-well cell culture plates at 3x10^3 cells/cm^2 in 2 ml of growth medium. To determine levels of cell senescence, β-galactosidase cell staining was performed on day 7 at passages 5 and 15. Beta-galactosidase cell staining was performed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technologies, Danvers, MA, USA) following the manufacturer's instructions.

β-Galactosidase staining is the characteristic staining of senescent cells, an arrested state in which the cell remains viable, and is not found in presenescent, quiescent, or immortal cells. Cells with the bright blue β-Galactosidase cytoplasmic staining were observed and counted under an inverted microscope (Eclipse Ti-S, Nikon, Tokyo, Japan) at 20x magnification, and also the total number of cells in the same field were counted. Each sample was counted randomly 5 times, and subsequently the percentages of β-galactosidase stained cells were calculated. The number of samples was 3 samples per group at each investigation time (n=4).

Cytogenetic analysis

Human BFP-ADSCs and DPSCs at passage 15 were seeded in T25 flasks (Corning Life Science, New York, NY, USA) at 3x10^3 cells/cm^2, grown in growth medium to 70.0% confluence, and sent to the Human Genetics Unit, Pathology Department, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand for karyotype analysis.

Alkaline phosphatase activity assay

Human BFP-ADSCs and DPSCs at passages 5, 10, and 15 were seeded at 5x10^3 cells/cm^2 in a 6-well cell culture plates and cultured in an osteogenic medium for 14 days. Then the cells were lysed in 1.0% Triton X-100 in PBS and centrifuged at 400 g for 5 min (Refrigerated Centrifuge, Labofuge 400 R) to obtain total protein lysis solutions and cell pellets. The amount of protein content in the total protein lysis solutions was measured using a Bio-rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.[27,29] After that, alkaline phosphatase (ALP) activity in the total protein lysis solutions was measured using an Alkaline Phosphatase Yellow Liquid Substrate for ELISA kit (Sigma-Aldrich) following the manufacturer's instructions and the optical density was measured at 405 nm absorbance in duplicate using a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The levels of ALP activity were standardized by the amount of total protein content in each sample and reported as micro-molar p-nitrophenol per milligram proteins (μM/mg proteins).[27-29]

Alkaline phosphatase and von kossa double staining

Human BFP-ADSCs and hDPSCs at passages 5 and 15 were seeded at 5x10^3 cells/cm^2 in 6-well cell culture plates and cultured in an osteogenic medium for 21 days. Then the cells were fixed in 4.0% paraformaldehyde (Sigma-Aldrich) and stained with an Alkaline Phosphatase Detection Kit (with fast red-violet red staining at passage 5 and fast blue RR blue staining at passage 15) (EMD Millipore, Darmstadt, Germany) following the manufacturer's instructions. Von Kossa staining was performed using 5.0% silver nitrate (Sigma-Aldrich) and counterstained with either hematoxylin or neutral red for either red or blue ALP staining, respectively, following standard protocols.[27, 28]

Statistical analysis

Data are presented as mean±standard deviation. The different results of each group of hBFP-ADSCs and hDPSCs at different time points were analyzed by repeated measures ANOVA. At each time point, the differences

among passages were analyzed by one-way analysis of variance (ANOVA) and compared multiple times with Scheffe or Dunnett T3 test as appropriate. Independent T-test was used to test the differences of doubling time and cell senescence between cell types (hBFP-ADSCs and hDPSCs) at each passage. Significance was set at p-value<0.050. Data were analyzed using SPSS statistical software for PC (SPSS, v.16.0, Chicago, IL USA).

Results

Cell culture

From each donor, the average amount of harvested buccal fat pad was 595.4±80 mg, and pulpal tissue was derived from 2 wisdom teeth, the upper and lower third molars. Cell yields from hBFP-ADSCs at the primary passage were $1.9x10^6±2.4x10^5$ cells ($3.2x10^5±9.9x10^3$ cells/100 mg of hBFP-ADSCs) and the number of hDPSCs from the 2 molars was $2.27x10^6±4.8x10^5$ cells ($1.14x10^6±2.4x10^5$ cells/tooth) (Figure 1).

Cell morphology and cell numbers

Human BFP-ADSCs and hDPSCs exhibited different cell morphologies. Human BFP-ADSCs were elongated spindle-shaped cells that spread and extended cytoplasmic processes on cell culture surfaces creating multiple cytoplasmic processes and intercellular contacts, while the hDPSCs were relatively small and compact fibroblast-like cells. The differences in cell sizes were reflected by the different number of cells in the T75 cell culture flasks at 80.0% confluence, in which the average number of cells at

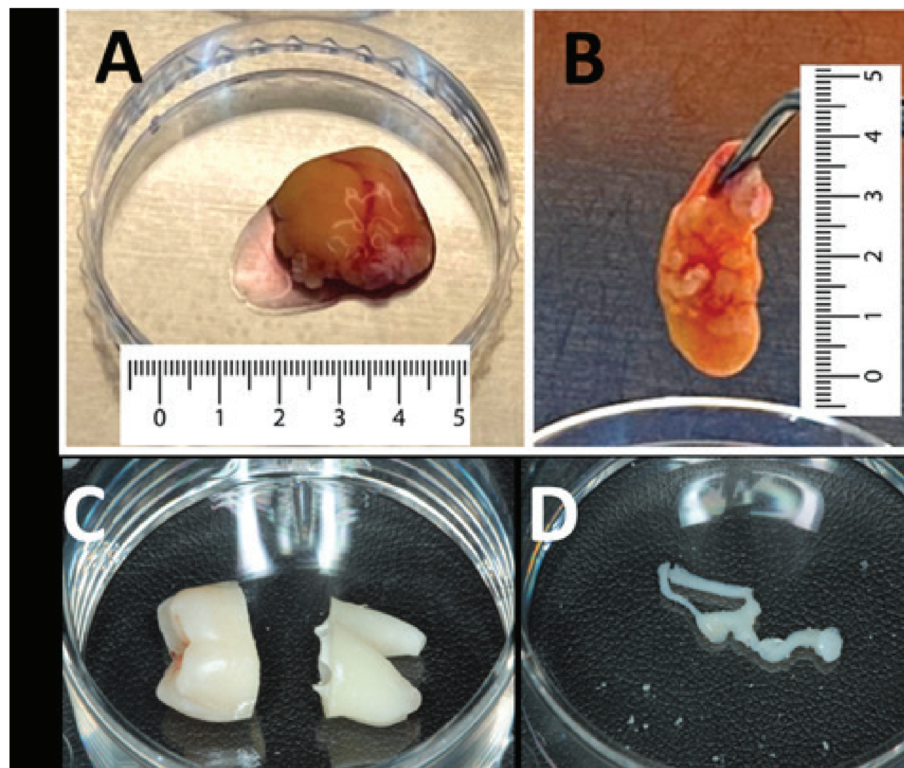

Figure 1 Buccal fat pad (BFP) from the buccal vestibule of the upper third molar (A, B), pulpal tissue from the third molar (C, D).

passages 1- 5 of hBFP-ADSCs was $5.36x10^5 \pm 2.33x10^5$ cells/T75 flask ($7.15x10^3$ cells/cm^2) while for the hDPSCs was $1.36x10^6 \pm 1.77x10^5$ cells/T75 flask ($18.20x10^3$ cells/cm^2). The number of hDPSCs was approximately 2.5 times higher than the hBFP-ADSCs.

Cell growth

Human DPSCs initially exhibited a high cell growth rate but which markedly decreased with subsequent cell passaging or cell expansion, while the hBFP-ADSCs demonstrated low and steady cell growth throughout the *in vitro* cell expansion. The cell proliferation capacity of the hDPSCs at passage 5 was highest, followed by the hDPSCs at passage 15 and the hBFP-ADSCs at passages 5 and 15 (p-value<0.010). The growth of hBFP-ADSCs at both passage 15s was significantly less than at passage 5 (p-value<0.010) but was much less than the reduction of hDPSCs between passages 5 and 15 (p-value<0.010) (Figure 2).

Cell doubling time

Cell doubling time (DT) showed different cell growth patterns between hBFP-ADSCs and hDPSCs and indicating the adverse effects of cell expansion on cell growth. The DT of hDPSCs at passages 5 and 15 was lower than the DT of hBFP-ADSCs at the same passages and significantly lower in passage 5 (p-value<0.050). The DT of hDPSCs at passage 15 was significantly higher than at passage 5 (p-value<0.010), but the DT of hBFP-ADSCs at passages 5 and 15 were not significantly different (p-value>0.050) (Figure 3).

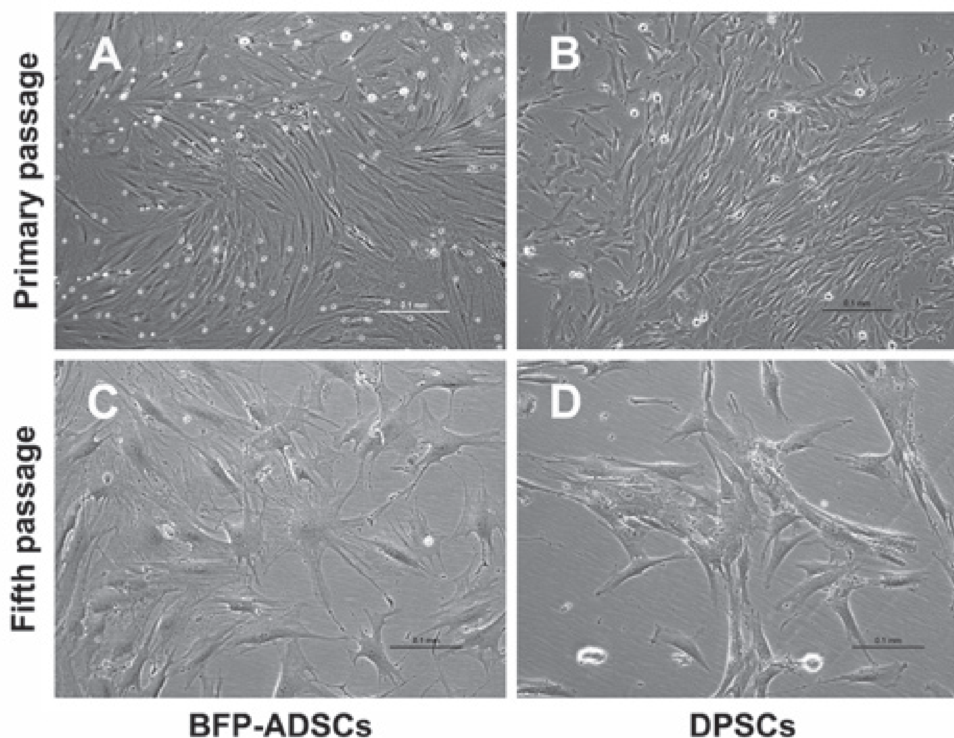

Figure 2 Exhibiting the cell morphology of buccal fat pad adipose-derived stem cells (BFP-ADSCs) (A, C) and dental pulp stem cells (DPSCs) (B, D) at the primary (A, B) and the fifth (C, D) passage.

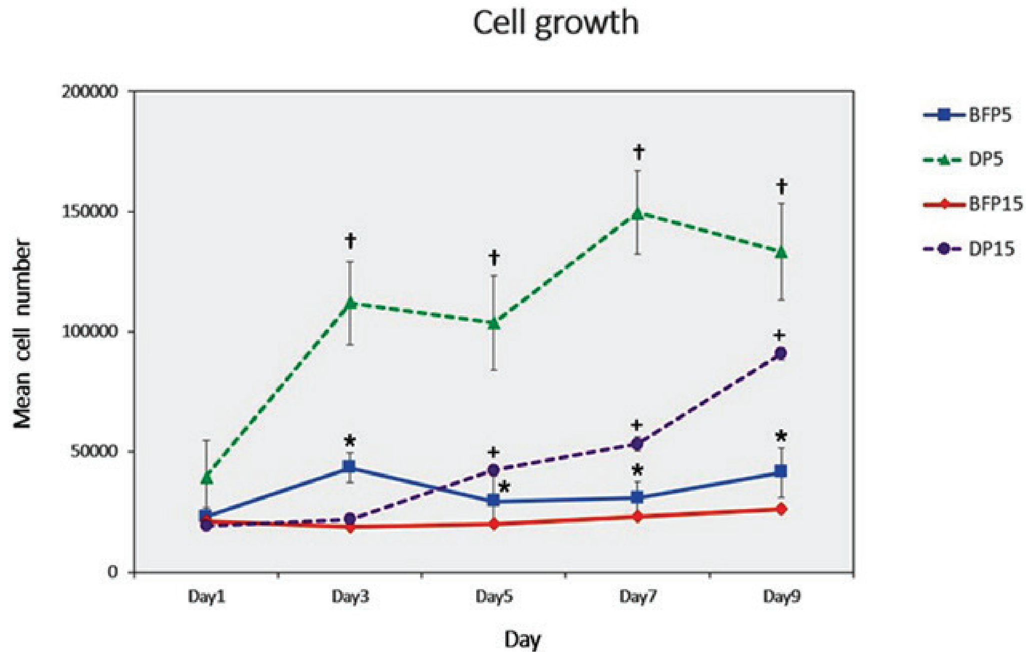

Figure 3 Cell growth of buccal fat pad adipose-derived stem cells (BFP-ADSCs) and dental pulp stem cells (DPSCs) at passages 5 and 15. The symbol (†) represents the highest levels of cell growth, (+) significant differences of at passage 5 and BFP-ADSCs at passages 5 and 15, and (*) significantly higher levels of cell growth of BFP-ADSCs at passages 5 than 15 (n=4, mean±S.D., p-value<0.050).

Cell senescence

Cell senescence was shown by blue cytoplasmic staining of beta-galactosidase. Most of the cells at passage 15 of both hBFP-ADSCs and hDPSCs exhibited intense blue cytoplasmic staining, while a few cells in either group at passage 5 showed light blue staining. The numbers of beta-galactosidase stained cells of both passage 15s were significantly higher (p-value<0.010) than passage 5, but the number of positively stained cells between these two groups were not significantly different (p-value>0.050)

Karyotype analysis

The karyotype anlaysis of the cells found all 20 G-banded metaphase cells of hBFP-ADSCs and hDPSCs at passage were normal female karyotype (Figure 4).

Alkaline phosphatase activity

The osteoblastic differentiation potential of both hBFP-ADSCs and hDPSCs was high during passages 5 to 10 and then markedly decreased at passage 15 that during this time *in vitro* cell expansion was extensive. The hBFP-ADSCs showed the highest level of ALP activity at passage 5, which was decreasing by passage 10 to the significantlly lowest level at passage 15 (p-value<0.010). The hDPSCs presented the same pattern with lower activity than the hBFP-ADSCs at passages 5 and 15. The ALP activity of the hDPSCs slightly increased at passage 10 and was higher than the hBFP-ADSCs and then decreased to the lowest level at passage 15 which was signicantly less than the other passages and the hBFP-ADSCs (p-value<0.010). (Figure 5).

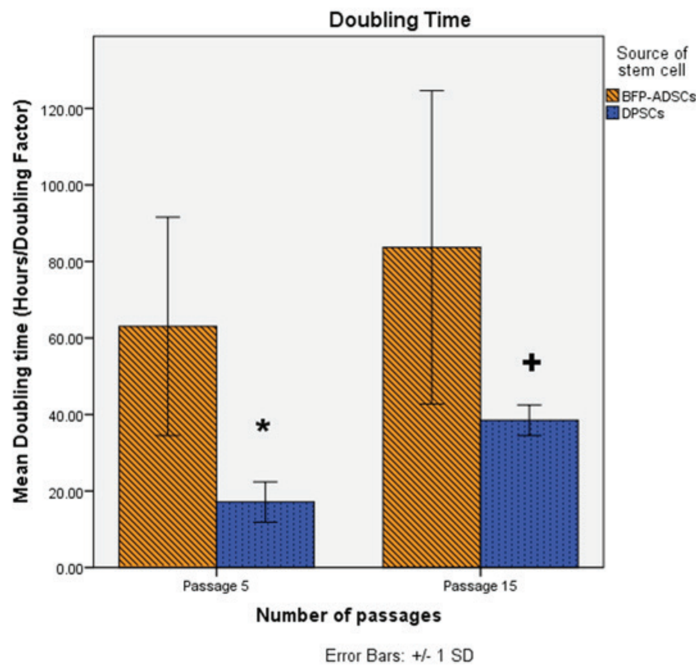

Figure 4 Doubling time of buccal fat pad adipose-derived stem cells (BFP-ADSCs) and dental pulp stem cells (DPSCs) at passages 5 and 15. The symbol (*) represents significant differences between groups at the same passage and (+) significant differences of the same group at different passages (n=4, mean±S.D., p-value<0.050).

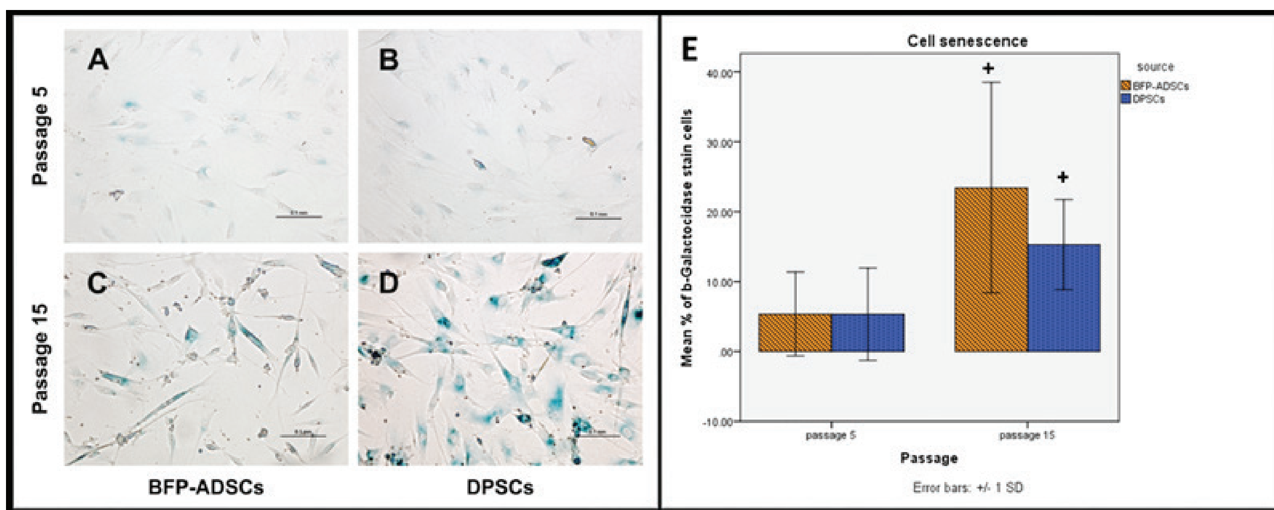

Figure 5 Presenting cell senescence beta-galactosidase staining of buccal fat pad adipose-derived stem cells (BFP-ADSCs) (A, C) and dental pulp stem cells (DPSCs) (B, D) at passages 5 (A and B) and 15 (C and D), and a diagram of percentages of beta-galactosidase-stained cells (E). The arrows indicate bright blue cytoplasmic staining of beta-galactosidase on the cytoplasm of the senescent cells. The symbol (+) represents significant differences of the same group at different passages (n=4, mean±S.D., p-value<0.050).

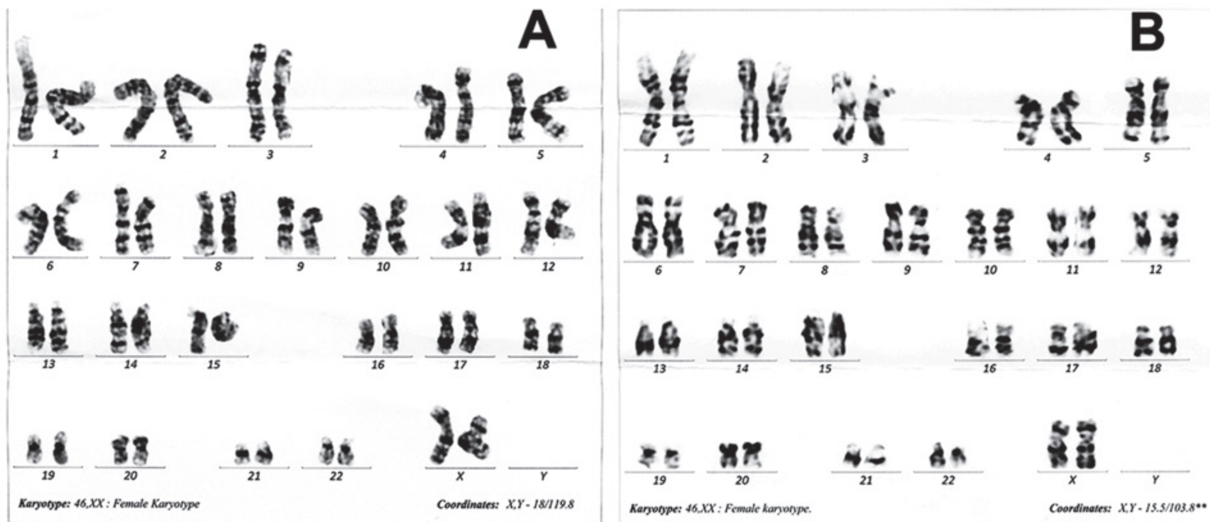

Figure 6 Karyotype analysis of buccal fat pad adipose-derived stem cells (BFP-ADSCs) (A) and dental pulp stem cells (DPSCs) (B) at passage 15. The analyses exhibit normal female karyotypes.

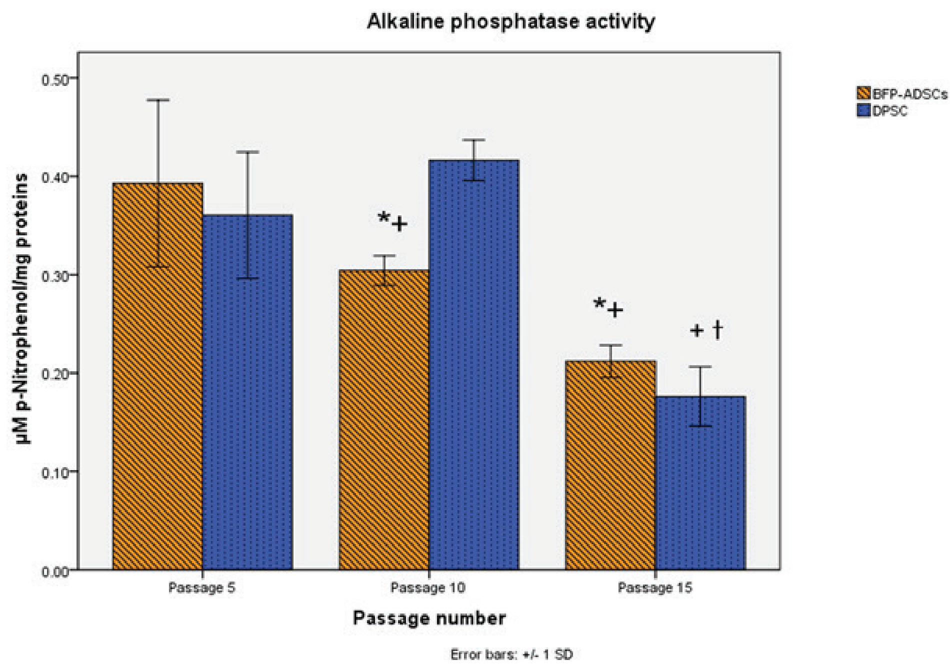

Figure 7 Alkaline phosphatase (ALP) activity of buccal fat pad adipose-derived stem cells (BFP-ADSCs) and dental pulp stem cells (DPSCs) at passages 5, 10, and 15. The symbol (*) represents significant differences between groups at the same passage, (+) significant differences of the same group at different passages, and (†) the lowest levels of ALP activity (n=4, mean±S.D., p-value<0.050).

Alkaline phosphatase and von kossa staining

The hBFP-ADSCs and hDPSCs were able to differentiate into mature osteoblasts and mineralized extracellular matrices and the osteogenic differentiation potential was markedly decreased at passage 15. Cells from both sources exhibited ALP staining on the cell surface, in pink at passage 5 and blue at passage 15, and von Kossa staining on the extracellular matrix showed dark brown to black staining. The amount and intensity of ALP and von Kossa staining at passage 15 were markedly lower than at passage 5. The staining demonstrated decreasing *in vitro* mineralization at passage 15 (Figure 6).

Discussion

Adipose stem cells have shown high bone regeneration capacity in craniofacial bones.[5,13] BFP-ADSCs have demonstrated the same osteogenic differentiation capacity as stem cells isolated from body adipose tissue, bone marrow, and unrestricted somatic stem cells[30] and would be a potential alternative to autologous bone grafting for oral and maxillofacial bone reconstruction.[10] Harvesting of the buccal fat pad is simple for an oral and maxillofacial surgeon when compared to harvesting body fat and can be done with patients of any age. We found that hBFP-ADSCs and hDPSCs exhibited different cell morphologies and cell growth kinetics corresponding to previous studies[31,32] which reported that hBFP-ADSCs were elongated spindle-shaped cells with a steady low proliferation rate, while hDPSCs were compact fibroblast-like cells with high and unstable proliferation capacity. The highest growth rate of hDPSCs was found in passage 5 and markedly decreased following cell expansion in passage 15. However, the osteogenic

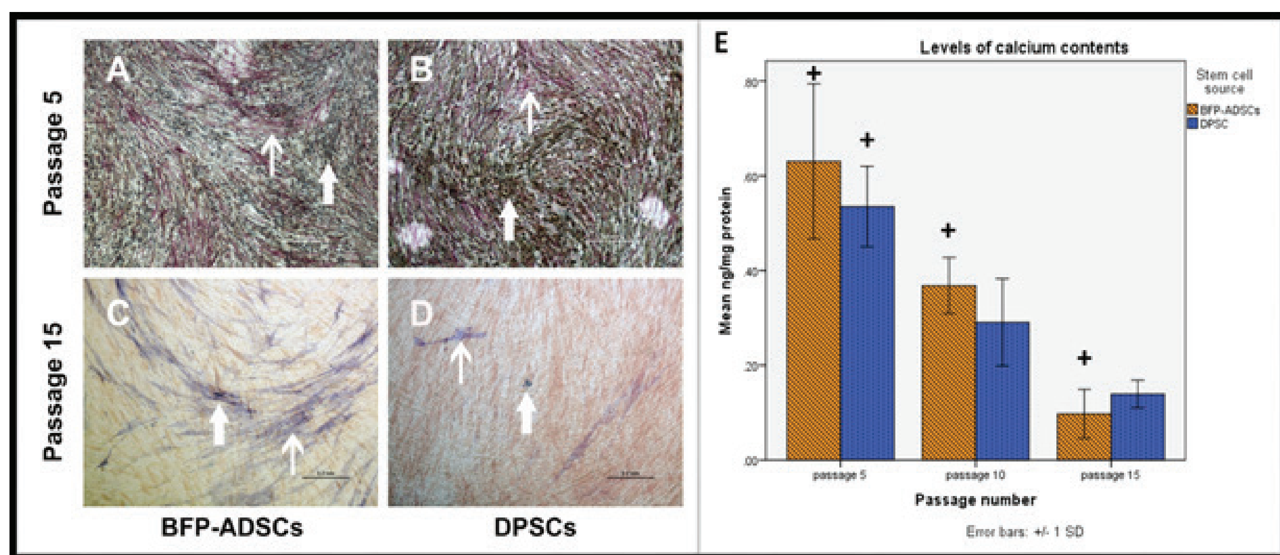

Figure 8 Alkaline phosphatase (ALP) and Von Kossa double staining of buccal fat pad adipose-derived stem cells (BFP-ADSCs) (A, C) and dental pulp stem cells (DPSCs) (B, D) at passages 5 and 15, and levels of calcium contents (E). The thin arrows indicate cytoplasmic staining of ALP in pink with hematoxylin counterstaining in blue (A, B) and ALP in blue with neural red in red counterstains (C, D). The thick arrows indicate dark brown to black von Kossa staining on mineralized extracellular matrices and nodules (A-D). The symbol (+) represents significant differences of the same group at different passages (n=4, mean±S.D., p-value<0.050).

differentiation potentials of hBFP-ADSCs and hDPSCs were comparable as demonstrated by similar levels of ALP activity and in vitro mineralization markers for early and late osteoblastic differentiation stages respectively.[33] These findings are different from previous reports which found that the growth and osteogenic differentiation potential of hSC-ADSCs were higher than in hDPSCs.[34,35] The current study compared hBFP-ADSCs and hDPSCs from three same patient-matched cases. Although the number of cases in this study was small, individual variation among the donors could be minimized following which we obtained homogenous results. Different cell types and morphologies might have been related to cell behaviors as seen in cell growth and doubling time, and affected the spreading of cytoplasmic processes on cell culture surfaces indicating that focal contact signal transduction lead to different cell behaviors.[36] Other studies have reported that the local environment in different stem cell depots such as vascularization and local cell types also affected cell behaviors.[31,37]

Following extensive cell expansion, cell growth and differentiation potential decreased and activated cell death. The marked decrease of ALP and von Kossa staining following cell expansion indicated significant adverse effects of cell expansion on mineralization and bone formation capacity.[21,33] In normal somatic cells, the expanded cells are normally able to maintain stable karyotypes. In this study, at passage 15, there was an increase of b-galactosidase staining, senescence and aging-associated enzymes that indicated cell senescence, but the cells were able to maintain stable karyotypes with an absence of malignant transformation.[20,22] Thus, it could be inferred that the transplanted hBFP-ADSCs and hDPSCs exhibited genetic stability and limited life span and should be safe for transplantation. For optimum growth and regeneration capacity of the transplanted cells, in vitro cell expansion should not be done for more than 5 passages. The adverse effects of cell expansion underline the importance of tissue availability from stem cell sources.

The accessibility and availability of hBFP-ADSCs are superior to dental pulp and other dental tissues because they are not dependent on age and not quantity limited. Based on our findings, 1 gm/or 1 ml of buccal fat pad could generate $3.2x10^6$ hBFP-ADSCs/ml with availability of up to 3-5 ml for stem cell isolation 10, with estimated cell yield of up to $9\text{-}15x10^6$ cells ($3.2x10^6$ cells x 3-5 ml). A previous study reported that maxillary sinus augmentation would require around $2x10^6$ mesenchymal stem cells to be delivered with biomaterial for grafting.[40] The average sizes of the buccal fat pads used in previous studies for intra-oral reconstructions were 9.3 g and 9.6 ml which were a large amount for stem cells.[38,39] In contrast, dental pulp requires tooth extraction and is limited in availability, particularly in advanced-age patients due to obliterated pulp chambers.[16,19] Our findings support the hypothesis that a single buccal fat pad could provide a sufficient quantity of hBFP-ADSCs for large alveolar bone defect reconstruction. Although other studies have reported that hDPSCs provided high cell proliferation that compensated for its limited availability of pulpal tissue, they also said that the adverse effects of multiple cell expansions should be considered.[20,21] Harvesting the buccal fat pad is simple and causes less pain and donor site morbidity[3,4] than bone block graft harvesting, and is superior to dental tissue in terms of availability.

Conclusion

Taken together, the accessibility and availability of hBFP-ADSCs through the oral cavity confirms the potential clinical application of hBFP-ADSCs transplantation in older-aged patients whose third molars are not available and whose health status might be compromised and limit the harvesting of subcutaneous fat. Based on a less-invasive procedure, stable cell growth, and good capability for osteogenic differentiation, hBFP-ADSCs transplantation

is a potential alternative to autologous bone grafting in patients with large and severe bone defects, particularly in cases requiring vertical augmentation. Human BFP-ADSCs transplantation would improve the efficiency and reliability of large augmentations in which osteogenesis is required to accomplish bone regeneration.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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