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Original Article

Proliferation and mineralization ability of dental pulp cells derived from primary and permanent teeth

Zheng Guan[1], Songtao Shi[2], and Suttatip Kamolmatyakul[1]*

[1] *Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.*

[2] *University of Southern California, Center for Craniofacial Molecular Biology, Los Angeles, California, United States of America*

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Abstract

The aims of this study were to compare the proliferation and mineralization ability of CFU-F selected dental pulp cells derived from primary and permanent teeth. Those cells were isolated by enzyme digestion and analyzed for their colony-forming capacity. The cell proliferation was measured by the MTT assay on day 1, day 7, and day14. Alizarin Red S staining was used to detect mineralized nodule formation of the cells on day 7, 14, 21, and 28. Proliferation of CFU-F selected pulp cells from primary teeth was significantly higher than that of CFU-F selected pulp cells from permanent teeth in all periods of the experiment. Upon cultured cells in mineralization inducing media, the mineralized nodules appeared as early as day 14 in CFU-F selected pulp cells from primary teeth and MG-63, whereas those of CFU-F selected pulp cells from permanent teeth can be found at day 21. On day 21 and day 28, the mineralized nodules of the CFU-F selected pulp cells from the primary teeth group were more than those in the CFU-F selected pulp cells from the permanent teeth group. Mineralized nodule formation in the CFU-F selected pulp cells from the permanent teeth group appeared later and were less than those of CFU-F selected pulp cells from primary teeth. However, mineralized nodules in CFU-F selected pulp cells from the permanent teeth group increased very fast after their appearance. Those results suggest that CFU-F selected pulp cells from primary teeth had a higher proliferation rate and mineralization rate when compared to CFU-F selected pulp cells from permanent teeth.

Keywords: CFU-F (colony-forming efficiency), dental pulp cells, primary teeth, permanent teeth, proliferation, mineralization

1. Introduction

Recently, the research of tissue engineering has made great progress. Stem cells are key elements in tissue engineering. Previous studies demonstrated that human dental pulp stem cells (DPSCs) (Gronthos *et al.,* 2000) and those stem cells in human exfoliated deciduous teeth (SHED) (Miura *et al*., 2003) possess properties of high proliferative potential, self-renewal capacity, and multi-lineage differentiation, like a novel adult stem cell. Liu *et al*. (2006) summarized the DPSCs isolation protocol and the prospect of DPSCs in tissue regeneration. The SHED was also suggested as an ideal source of stem cells for restoration of damaged tooth structure, bone regeneration, and therapy of neural tissue injury or degenerative disease (Miura *et al*., 2003).

The golden standard in stem cell transplantation is the use of an autologous stem cell source. Homologous stem cell transplantations can cause pathogen transmission and need immunosuppression as long as any other tissue or organ transplantation procedure (Graziano *et al.,* 2008).

* Corresponding author.
Email address: suttatip.k@psu.ac.th

DPSCs and SHED provide opportunity for patients use their own stem cells, and those two cell lines can be obtained from a unique organ; the tooth. A previous study showed that the purified SHED possessed a higher proliferation rate than purified DPSCs (Miura *et al*., 2003). The purified stem cells should be obtained from three or four different teeth pulp tissues, and the purification step involved immuno-magnetic bead sorting including the use of several antigens, for example, STRO-1 and $CD146^+$ (Shi and Gronthos, 2003). DPSCs are a heterogeneous population of postnatal stem cells akin to BMMSCs (Liu *et al.,* 2006). In fact, DPSCs and SHED can be isolated by their ability to generate clonogenic adherent cell clusters when plated under the same growth conditions as described for BMSSCs (Shi *et al.,* 2005). For colony-forming efficiency (CFU-F) of selected pulp cells, about 82 percent, were represented in STRO-1 positive, being 6-fold greater than in non–CFU-F selected pulp cells, whereas 96% of CFU-F cells are present in the $CD146^+$ population, being 7-fold greater than in non–CFU-F selected pulp cells (Shi and Gronthos, 2003). In order to simplify the process and save costs of cell isolation procedure, CFU-F selected pulp cells can possibly be used instead of purified SHED and DPSCs. Therefore, before these unique cell resources can be used for further research and potential clinical application, we need to find out which ones possess better functions according to their properties. This study is designed to observe the proliferation and mineralization ability of CFU-F selected pulp cells derived from human primary and permanent dental teeth pulp.

2. Materials and Methods

2.1 Cell culture

Primary and permanent teeth were collected under the approved guideline of the Ethical Committee of Prince of Songkla University (521.1.03/629. Human primary exfoliated teeth were collected from 6 to 12 year old children (n=6). Informed consent was obtained from the parents. Permanent teeth were obtained from adult donors ($\leq$ 29 years old, n=6). Impacted third molars and bicuspids were extracted due to orthodontic considerations (Hahn *et al.,* 1989). All of these teeth contained a normal healthy pulp and were confirmed by clinical and radiographic examination (Cohen *et al.,* 1985).

Dental pulp cells were isolated as described by Gronthos *et al.* (2000). Tooth surfaces were cleaned with 70% alcohol and then cuts around the cementum-enamel junction were by using sterilized dental fissure burs to reveal the pulp chamber. The pulp tissue was gently separated and minced. The minced pulp tissues were digested in a mixture of 3 mg/ml collagenase type I and 4 mg/ml dispase (Dissolved in PBS, Sigma, St. Louis, Mo., U.S.A.) for 30-60 min in a 37°C water-bath. Cell suspensions were obtained by passing the digested tissues through a 70–μm cell strainer (Becton/Dickinson, Franklin Lakes, N.J., U.S.A.). Single cell suspensions were seeded in 100 mm culture plates (Nunc, Denmark) containing DMEM (Life Technologies/GIBCO BRL) supplemented with 20% fetal bovine serum (FBS, Biochrom AG, Germany), 2 mM L-glutamine (Gibco Invitrogen, USA), 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin and 100 μg/ml kanamycin, and maintained under 5% CO_2 at 37°C. Those cells were allowed to grow for 10 to 12 days, and then were collected by assessing their colony-forming efficiency (CFU-F) (Shi *et al.,* 2005). Cells aggregated in groups of less than 50 cells were scratched from the bottom of the plate and removed by phosphate buffered saline (PBS). The other colonies ($\geq$ 50 cells) were transferred to T-75 cultural flasks (TPP, Switzerland) and were cultured up to 70-80% confluence, and then they were passaged at 1:3 ratios for experiment or storage. Cells at passage 3 were used in the experiments.

2.2 Determined the cell proliferation by MTT assay

CFU-F selected pulp cells from primary teeth (n=6) and CFU-F selected pulp cells from permanent teeth (n=6) were seeded at the density of 3×10^3 cells/well in 96-well-plates (Nunc). At least three wells without cells served as a control for the minimum absorbance. After 24 hours, 7 days, and 14 days, the cell proliferation was measured by a MTT assay modified from Mosmann *et al.* (1983). Absorbance of the colored solution was measured at wavelength 572 nm by a plate reader (Biotrak II, Amersham Biosciences). The viabilities of each cell line were calculated according to the following formula (Machado *et al*., 2007):

Cell viability (%)
= 100 × measurement of day 14/ measurement of day 1,
= 100 × measurement of day 7/ measurement of day 1,
= 100 × measurement of day 14/ measurement of day 7.
Experiments were confirmed in triplicate.

2.3 Measured mineralized nodule formation by Alizarin Red S staining

In order to determine the differentiation ability of DPSC and SHED, the cultural condition of mineralized nodule formation was established by Tsukamato *et al.* (1992). CFU-F selected pulp cells from primary teeth, CFU-F selected pulp cells from permanent teeth, gingival fibroblast (served as the negative control), and MG-63 (served as the positive control) were seeded at the density of 2×10^4 cells /well in 24-well-plates (n=6). Those cells were cultured with normal growth media until reaching confluence. Then the conditioning media containing 1 ml DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma), 100 μM L-ascorbic acid 2-phosphate (Sigma), 2 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 U/ml mycostatin, and 100 μg/ml kanamycin were added to each well. The plates were cultured at 37°C in an incubator setting in a humidified atmosphere of 5% CO_2. The cells within the normal media were set as the controls. The media

were changed every two days. Detecting of mineralization during cell differentiation was performed at day 7, 14, 21, and 28. The mineralized nodules were stained using Alizarin Red S (Nacalai Tesque Inc.) (Stanford *et al.,* 1995) and observed under a light microscope (Inverted Nikon TS 100E). The results were presented by descriptive analysis (Gay *et al.*, 2007; Vitale-Brovarone *et al*., 2007; Huang *et al*., 2008). Experiments were confirmed in triplicate.

2.4 Data analysis

Statistical analyses were performed by using SPSS software (Version 16.0, Standard Software Package Inc., U.S.A.). The data of proliferation result was presented as the mean ± SD. Differences among groups were analyzed using the Student's t-test. Significant differences were set at 95% confidence. A mineralization study was presented by descriptive analysis.

3. Results

3.1 Cell proliferation

As shown in Table 1 and Figure 1, the optical density (OD) volume is not more than 1 in CFU-F selected pulp cells from the primary teeth group and the permanent teeth group, therefore those two cell lines were in exponential growth phase. There was no significant difference among the different cell groups on day 1. On day 7, the OD of CFU-F selected pulp cells from primary teeth was found to be significantly higher than that of CFU-F selected pulp cells from permanent teeth. On day 14, the OD of CFU-F selected pulp cells from primary teeth was still markedly higher than that of CFU-F selected pulp cells from permanent teeth. Moreover, these two cell lines proliferated fast in the first 7 days, but the proliferation of those cells slowed down after 7 days. CFU-F selected pulp cells from primary teeth proliferated significantly higher than CFU-F selected pulp cells from permanent teeth from 1 to 7 days and from 1 to 14 days. After day 7, CFU-F selected pulp cells from primary teeth proliferated slower than CFU-F selected pulp cells from permanent teeth (Figure 2).

3.2 Mineralized nodule formation

There was no nodule formation on day 7 in all groups (Figure 3). On day 14, some mineralized nodules could be found in the test groups of CFU-F selected pulp cells from primary teeth and MG-63. In contrast, we could not find any mineralized nodules surrounding the cells in the test groups of CFU-F selected pulp cells from permanent teeth and gingival fibroblasts (Figure 4). On day 21, the nodules were present in every test group. Many more mineralized nodules could be observed both in the CFU-F selected pulp cells from primary teeth and the MG-63 group compared to the CFU-F selected pulp cells from the permanent teeth group.

Table 1. CFU-F selected dental pulp cells from primary and permanent teeth proliferation detected by MTT assay.

	SHED	DPSC
Day 1	0.201±0.061	0.145±0.042
Day 7	0.586±0.408*	0.308±0.116*
Day 14	0.815±0.335*	0.495±0.182*

Data was analyzed by Student's t-test, and the statistical significance was accepted at the 0.05 confidence level (*P<0.01).

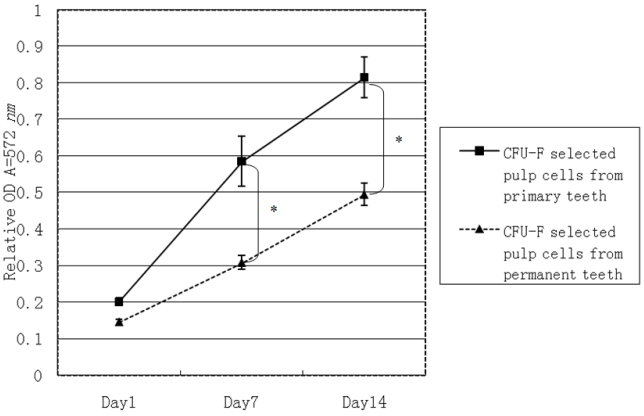

Figure 1. CFU-F selected dental pulp cells from primary and permanent teeth proliferation detected by MTT assay: OD (A=572 nm) was expressed as a measure of cell proliferation on days 1, 7 and 14, n=6. Error bars represent means ± SD. Data was analyzed by Student's t-test, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.01)

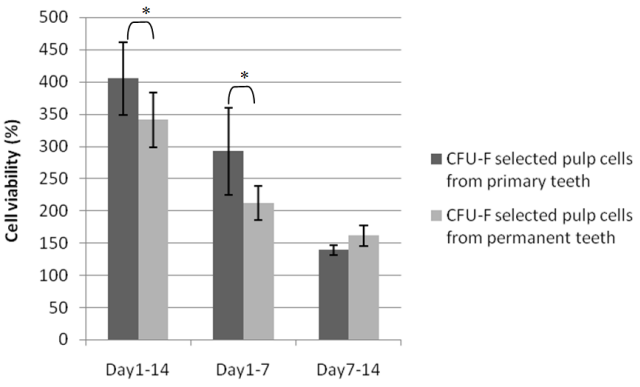

Figure 2. Cell viabilities of CFU-F selected dental pulp cells from primary and permanent teeth. Viabilities of CFU-F selected dental pulp cells increased from 1 to 7 days, 7 to 14 days and 1 to 14 days were presented, n=6. Error bars represent means ± SD. Data was analyzed by Student's t-test, and the statistical significance was accepted at the 0.05 confidence level. (* P<0.05)

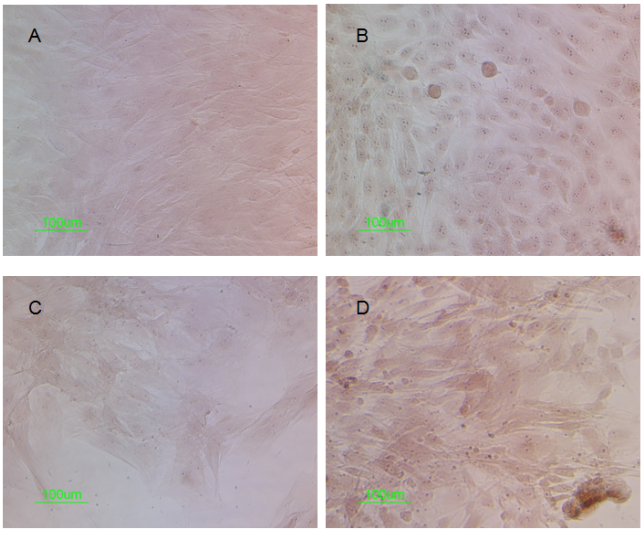

Figure 3. Mineralized nodule formation detected by Alizarin Red S staining on day 7. A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

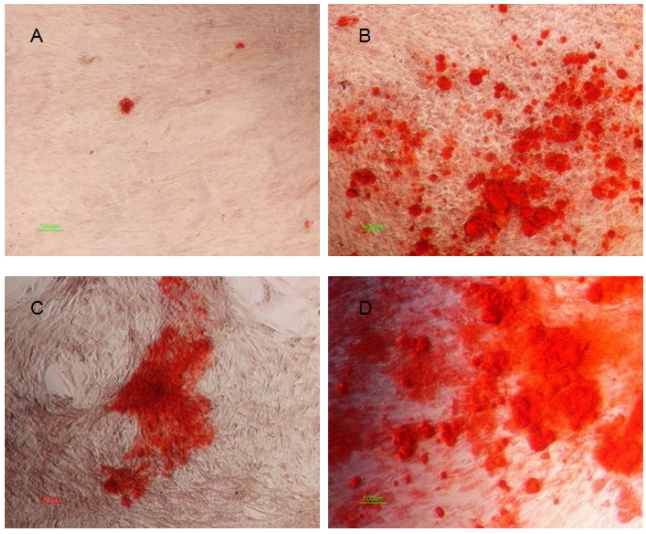

Figure 5. Mineralized nodule formation detected by Alizarin Red S staining on day 21. A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

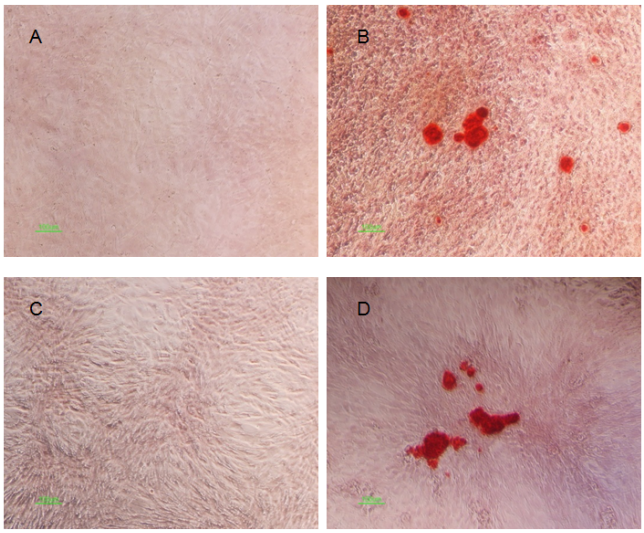

Figure 4. Mineralized nodule formation detected by Alizarin Red S staining on day 14. A. Gingival fibroblasts; B. MG-63; C. DPSC; D. SHED.

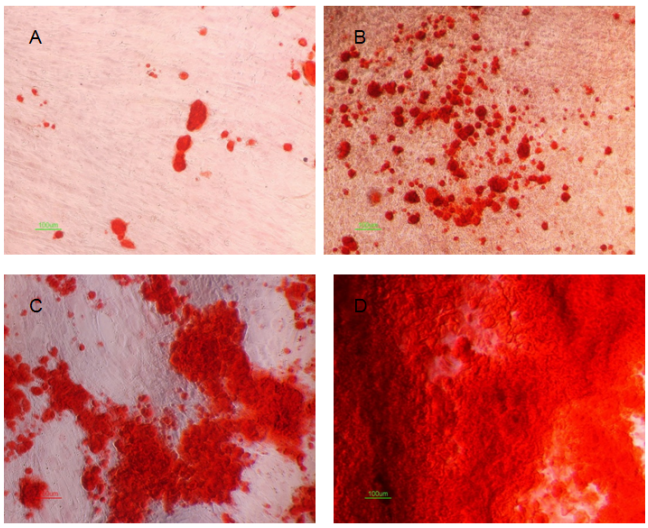

Figure 6. Mineralized nodule formation detected by Alizarin Red S staining on day 28.

In addition, few mineralized nodules could be found in the gingival fibroblast group (Figure 5). On day 28, a great number of mineralized nodules appeared in the test groups of CFU-F selected pulp cells from permanent teeth and CFU-F selected pulp cells from primary teeth, and mineralized nodules in the CFU-F selected pulp cells from primary teeth groups were still more than in the CFU-F selected pulp cells from the permanent teeth group. However, the circumstances for miner-alized nodule formation in the MG-63 test group were not different from day 21 (Figure 6).

4. Discussion and Conclusion

Langer and Vacanti (1993) reported that the most common approach for engineering biological substitutes is based on seeded cells, signal molecules, and polymer scaffolds. The presence of the unique populations, DPSC and SHED, has been reported (Gronthos *et al.,* 2000; Miura *et al*., 2003). Those two cell lines are capable of extensive proliferation. Previous studies showed that the proliferation of SHED is significantly higher than that of DPSC (Miura *et al*., 2003;

Galler *et al*., 2008). We compared the proliferation trends between CFU-F selected pulp cells from primary teeth and permanent teeth. Previous studies detected the cell proliferation trends by measuring their optical density (Schmalz *et al.,* 2001; Seda Tiğli *et al.,* 2007; Jue *et al.,* 2010). The result showed the CFU-F selected pulp cells from primary teeth proliferate significantly higher than those from permanent teeth. CFU-F selected pulp cells contain a high volume of stem cells (Shi and Gronthos, 2003). Hence, the CFU-F selected pulp cells revealed a similar proliferation ability as magnetic bead sorting purified SHED and DPSCs. Therefore, CFU-F selected pulp cells could possibly be served as the seeded cells in tissue engineering research, and it may save the cost of magnetic bead sorting.

Next, we examined the mineralization ability of CFU-F selected pulp cells from primary and permanent teeth. The osteosarcoma cell line, an osteoblast-like cell MG-63 (Billiau *et al.,* 1977), which can form mineralized nodules under osteogenic condition, was set as the positive control. The gingival fibroblasts were set as the negative control. Mineralized nodules can be detected by Alizarin Red S staining from 2 to 8 weeks in human dental pulp cell calcified culture, and the purified SHED and DPSCs possess a faster mineralization ability than the normal dental pulp cells (About *et al.,* 2000; Gronthos *et al.,* 2000; Alliot-Licht *et al.,* 2001; Miura *et al*., 2003). Our results showed that earlier nodule formation can be found at day 14 in the test groups of CFU-F selected pulp cells from primary teeth and at day 21 in the test groups of CFU-F selected pulp cells from permanent teeth. These results suggest that the CFU-F selected pulp cells are able to maintain the mineralization ability as well as SHED and DPSCs. However, mineralized nodules in the test groups of CFU-F selected pulp cells from primary teeth appeared earlier than those of CFU-F selected pulp cells from permanent teeth. There are many factors that affect the time of mineralization, such as the isolation method, seeding density, and donor age (Mauth *et al.,* 2007). Our results showed that the proliferation of CFU-F selected pulp cells from primary teeth were significantly higher than that of CFU-F selected pulp cells from permanent teeth. Therefore, it cost less time for CFU-F selected pulp cells from primary teeth than CFU-F selected pulp cells from permanent teeth to achieve the mineralization required density. Moreover, as shown from Figure 2, CFU-F selected pulp cells from permanent teeth proliferate more than CFU-F selected pulp cells from primary teeth, after 7 days. This implies that the mineralized nodules in the test group of CFU-F selected pulp cells from permanent teeth increased very fast after their appearance. The mineralized nodules appeared from day 14 in the test groups of the MG-63 cell line. Previous studies showed that MG-63 and the other human osteoblastic cell line, respectively displayed the mineralized nodules at the 12^{th} and the 14^{th} day (Robinson *et al*., 1997; Luo *et al*., 2001). Interestingly, few mineralized nodules appeared in test groups of gingival fibroblast. In general, there was no mineralized nodule formation when gingival fibroblasts were cultured in mineralized condition medium (Mukai *et al*., 1993). Cames *et al*. (1997) reported that the mineralized condition culture of gingival fibroblasts was able to form mineralized nodules and those mineralized nodule formations required an appropriate microenvironment (Yajima *et al*., 1984). KH_2PO_4 is a component of PBS (phosphate buffered saline) and its ability of enhancing mineralization was confirmed by the use of organic Na-β-glycerophosphate (Zhang *et al.,* 2006). In our study, cells were washed by PBS at least two times before fresh medium were added during the medium change procedure. KH_2PO_4 possibly remained. When it associated with mineralized condition medium, the mineralization of the test groups enhanced. This could be a possible reason for the mineralized nodules appearance in the test groups of gingival fibroblast in our study.

Importantly Shi and Gronthos (2003) summarized several stem cell markers which expressed in SHED and DPSCs. Only two to three antigens are usually used for stem cell purification. Therefore, those populations possibly selected by other stem cell makers could be lost. The use of CFU-F selected pulp cells can probably solve this problem. However, further studies are required to confirm this assumption. In conclusion, the present study demonstrated that CFU-F selected pulp cells from primary teeth possessed a better proliferation capability and earlier mineralization ability than CFU-F selected pulp cells from permanent teeth.

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