Original Article

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Effect of human exfoliated deciduous teeth stem cell-derived conditioned medium on human periodontal ligament stem cell proliferation and osteogenic differentiation

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Objectives: The aim of this study was to investigate the effect of stem cells from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) on human periodontal ligament stem cell (PDLSC) proliferation and osteogenic differentiation.

Materials and Methods: SHED-CM was collected after culturing for 48 h. The experimental SHED-CM groups were prepared by ultrafiltration together with diethyl ether precipitation to produce two different molecular weights fractions: D<3 and D3-10. PDLSCs were cultured with and without the SHED-CM fractions. To evaluate the effect of SHED-CM on PDLSCs proliferation and osteogenic differentiation, a CCK-8 assay and alizarin red S staining, respectively, was performed.

Results: SHED-CM-treated PDLSC demonstrated significantly increased cell proliferation from Days 4–8. In contrast, PDLSC calcified nodule formation was inhibited in the SHED-CM groups.

Conclusion: The results suggest that precipitated SHED-CM with less than 10 kDa molecular weight stimulated PDLSC proliferation and inhibited their osteogenic differentiation. These findings warrant further investigations to identify the peptides in SHED-CM to maximize PDLSC proliferation or osteogenic differentiation for future applications for bone regeneration.

Keywords: bone regeneration, conditioned medium, cell proliferation, mesenchymal stem cell, peptide

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Introduction

Cleft lip and palate is one of the most common congenital anomalies in the orofacial region with an incidence of 1.51 per 1000 live births in Thailand [1]. Cleft patients usually require various forms of treatment from birth to adulthood. Secondary alveolar bone grafting is one of the procedures used to repair the alveolar cleft defect. However, this procedure often causes pain, infection,

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or scars at the donor site [2]. To overcome these complications, stem cell therapy is of interest because it can reduce donor site morbidity. Several types of stem cells, such as umbilical cord stem cells [3], bone marrow mesenchymal stem cells (BMSCs) [4], and more recently, stem cells from human exfoliated deciduous teeth (SHED) have demonstrated osteogenic potential when implanted *in vitro* [5] and *in vivo* [6]. A similar amount of bone formation has been found when using either SHED or BMSCs in mice [7].

Because the procedure for obtaining the cells is also non-invasive, SHED are becoming a promising alternative source of cells for repairing bone in cleft patients. However, cell-based therapy has limitations, such as a low survival rate of the implanted stem cells [8, 9], tumorigenicity [10], and immune system incompatibility [11]. Therefore, further research and development in this area is still necessary.

The main therapeutic mechanism of stem cell transplantation is due to the molecules released into the conditioned medium of the stem cells [12]. This has led researchers to explore using the conditioned medium, also known as the cell-free method, to be another treatment option in tissue regeneration. The SHED-derived conditioned medium (SHED-CM) has been suggested to be used to treat neural disorders due to its neural regenerative effects [13-15]. The anti-inflammatory effects of SHED-CM have also been harnessed for treating osteoarticular diseases [16, 17], cardiopulmonary injuries [18], and hepatic disorders [19]. SHED-CM has been found to stimulate angiogenesis in dental pulp regeneration [20]. Moreover, SHED-CM has also been reported to have osteoinductive effects that facilitated bone regeneration in mice [21] and after implant placement in dog femurs [22]. In addition, SHED-CM increased the proliferation of several cell types, including Schwann cells [23] and pancreatic β-cells [24]. The bioactive molecules in CM that cause these effects could be nucleic acids, lipids, extracellular vesicles, proteins or peptides [25]. Among these molecules, peptides exhibit certain advantages over other molecules, including high potency of action, high target specificity, the ability to penetrate the cell membranes, and low accumulation in organs [26]. However, the specific effects of the peptides in SHED-CM that contribute to its many effects have not yet been fully explored.

Periodontal ligament stem cells (PDLSCs) exist in the perivascular space of the periodontium that is adjacent to the alveolar cleft site. PDLSCs can differentiate into various cell types, including osteoblasts, and previous *in vivo* and *in vitro* studies have confirmed osteogenic differentiation and bone regeneration in bone defects treated with PDLSCs [27]. Therefore, PDLSCs are another potential tool for repairing bone defects in cleft patients. Further understanding of how PDLC proliferation and differentiation can be optimized is needed to maximize their benefits in regenerative treatment. Thus, the aim of our study was to evaluate the effect of SHED-CM on PDLSC proliferation and osteogenic differentiation

Materials and Methods

Cell Culture

SHED from the sixth to eighth passages (P 6–8) obtained from the study by Gonmanee T *et al,* Department of Oral Biology, Faculty of Dentistry, Mahidol University (Bangkok, Thailand) (IRB 2014/041.2110) [28] and human PDLSCs from the sixth to eighth passages (P 6–8) from ScienCell Research Laboratories (Carlsbad, CA, USA) were used in this study.

Both groups of cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone, Fisher Scientific, Logan, UT) containing 10% Fetal Bovine Serum (FBS, Biochrome, Berlin, GY) and 1% Penicillin-Streptomycin antimicrobial agent (10,000 U/mL penicillin and 10,000 µg/mL

streptomycin, Gibco, Thermofisher Scientific) and incubated at 37°C, 5% CO_2 and constant humidity. The medium was changed every two days.

SHED-Derived Conditioned Medium (SHED-CM) Preparation

SHED from the sixth to eighth passages (P 6-8) were cultured as mentioned above. After achieving 80–90% confluence, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) before the serum-free DMEM (HyClone) was added. The conditioned medium was collected after 48 h and was filtered with a 0.2 µm syringe filter (Pall Laboratory, CA, USA) to eliminate debris or detached cells. This conditioned medium was designated as the SHED-derived conditioned medium (SHED-CM).

The SHED-CM was fractionated by ultrafiltration and diethyl ether precipitation. Briefly, the SHED-CM was concentrated using a centrifugal filter with a 10 kDa molecular weight cut-off (Amicon®, Merck Millipore Ltd., Darmstadt, Germany) by centrifugation at 5,000 g for 30 min. The fraction with molecular weight higher than 10 kDa was discarded.

The < 10 kDa fraction was repeatedly concentrated using a centrifugal filter with a 3 kDa molecular weight cut-off (Amicon®) by centrifugation at 5,000 g for 1 h, after which the SHED-CM was separated into two fractions: SHED-CM <3 kDa and SHED-CM 3–10 kDa.

Each SHED-CM fraction was separately precipitated by adding diethyl ether and left at -80°C to allow for peptide precipitation. After that, half of diethyl ether was poured out and fresh diethyl ether was added. This process was repeated until there was no additional peptide precipitation. Lastly, the diethyl ether was slowly evaporated at room temperature. The products from these procedures comprising the precipitated <3 kDa and 3–10 kDa SHED-CM fractions were designated as diethyl ether precipitated SHED-CM <3 kDa (D <3) and diethyl ether precipitated SHED-CM 3–10 kDa or (D 3–10), respectively. Both precipitated fractions were stored at -80°C until use.

Proliferation Assay

PDLSCs seeded were seeded at a density of 2,000 cells/well into a 96-well plate (Nunc, Thermo scientific) and incubated for 24 h to allow for cell attachment. The D <3 and D 3-10 fractions were weighed and dissolved in DMEM (Hyclone) containing 10% FBS (Biochrome) and 1% penicillin-streptomycin (Gibco) to obtain a final concentration of 0.1 mg/mL. Each of these prepared solutions of the SHED-CM fractions was filtered through a 0.2 µm filter (Pall Laboratory) before being used to treat the PDLSCs.

The control group was prepared by culturing PDLSCs with DMEM (Hyclone) containing 10% FBS (Biochrome) and 1% penicillin-streptomycin (Gibco), with this medium being changed every 2 d.

A cell counting kit-8 assay (CCK-8, Dojindo, Japan) was performed to evaluate PDLSC proliferation on Days 0, 2, 4, 6, and 8. According to the manufacturer's instructions, 10 µl CCK-8 reagent was added into each well before being incubated at 37°C for 2 h, followed by measuring the absorbance at 450 nm using a microplate reader (Bio-Tek, VT, USA). The experiment was done in triplicate.

Alizarin Red S Staining

PDLSCs were seeded at a density of 40,000 cells/well into a 24-well plate. After incubating for 24 h, the control group medium was replaced with Advance STEM osteogenic differentiation medium (HyClone) with 10% osteogenic supplement (HyClone) and 1% penicillin-streptomycin (Gibco) (osteogenic medium). In the experimental groups the PDLSCs were treated with osteogenic medium containing 0.1mg/mL of each SHED CM fraction (D <3 and D 3–10). Each group's medium was changed every 3 d.

On Day 28, calcified nodule formation was evaluated using alizarin red S staining. The culture medium was removed from each well and washed with PBS. Ice-cold 100% methanol was added and left at -20°C for 10 min for fixation. After rinsing each well twice with distilled water, 1% alizarin red S solution (Sigma-Aldrich, St. Louis, MO, USA) was added, then incubated at room temperature for 30 min, after which the remaining dye was rinsed out with distilled water.

The results were examined and photographed using an inverted light microscope (Nikon Eclipse TS100, Nikon Instruments, Melville, NY, USA) and a macro-zoom microscope (Olympus MVX10, Olympus Corporation, Tokyo, Japan). For quantification, the nodules in each well were dissolved with 10% cetylpyridinium chloride (Sigma). The absorbance at 570 nm was measured by using a microplate reader (Bio-Tek). The experiment was done in triplicate.

Statistical analysis

Statistical analyses were performed with the Statistical Package for Social Sciences software (SPSS v.21, Chicago, IL, USA). The quantitative data were expressed as mean ± standard deviation. The Kruskal-Wallis test was used to compare the differences between groups, and the Mann-Whitney U test was performed to compare two independent groups. The significance level was set at p-value <0.05.

Results

Effect of SHED-CM on PDLSC proliferation

The effect of SHED-CM on PDLSC proliferation as determined using CCK-8 assay is presented in Figure 1. On Day 0 and 2, there were no significant differences in PDLSC proliferation between the control and either experimental D<3 or D 3–10 groups. On Day 4, PDLSCs treated with D<3 demonstrated a significantly higher proliferation rate (13.4%) compared with the control group, but no significant difference were seen between the D3–10 and control groups. On Day 6, the D<3- and D 3–10-treated groups had higher proliferation rates of 15.2% and 7.6%, respectively compared with the control group, however, the difference between the two treatment

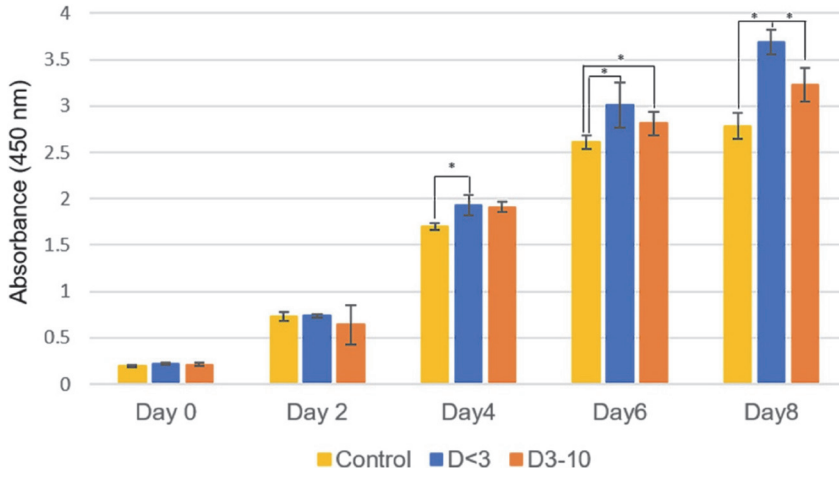

Figure 1 PDLSC proliferation induced by 0.1 mg/mL diethyl ether precipitated-SHED-CM <3 kDa (D<3) and 3–10 kDa (D 3-10) compared with the control group (DMEM containing 10% FBS and 1% penicillin-streptomycin). *Statistically significant difference at p<0.05 (n=5)

groups was not significant. On Day 8, cell proliferation increased by 32.5% in the D<3 group compared with the control group, and a 15.9% increase was seen in the D 3–10 group, with the proliferation rate in the D<3-treated group being significantly higher than the D 3–10 group by 14.3%. An overall gradual increase in PDLSC proliferation can be observed from Day 2–8 after treatment with diethyl ether precipitated SHED-CM compared with the control (Figure 1).

Effect of SHED-CM on PDLSC osteogenic differentiation of

To elucidate whether the D<3 and D3–10 fractions affected PDLSC osteogenic differentiation, alizarin red S staining was performed on Day 28, where it was observed that the characteristics of the alizarin red S staining in the control group were different from those of the SHED-CM treatment groups. In the control group, there was a homogenously spread mineral layer with small dark red nodules, whereas the experimental groups demonstrated dark red stained nodules surrounded by unstained areas (Figure 2A-C). The quantitative data also showed that the intensity of the extracted alizarin red S, which indicates the amount of calcium deposition in the D<3 and D3–10 groups were significantly lower than the control group, with no significant difference between the two SHED-CM groups (Figure 2D).

Discussion

Regenerative medicine of the bone and periodontal tissue would be beneficial in orthodontic treatment, especially in the repair of alveolar clefts. Previous reports indicated that SHED are a non-invasive and useful source for stem cell-based therapy [29]. However, due to their limitations, such as a low survival rate [8, 9], tumorigenicity [10], and immune incompatibility [11] of stem cell transplantation, studies have explored the use of bioactive molecules in the conditioned medium of stem cells instead of pure stem cell-based therapy. These studies have demonstrated remarkable effects on tissue regeneration through paracrine mechanisms [30, 31]. Thus, this form of cell-free therapy has been suggested as an alternate choice for regenerative medicine.

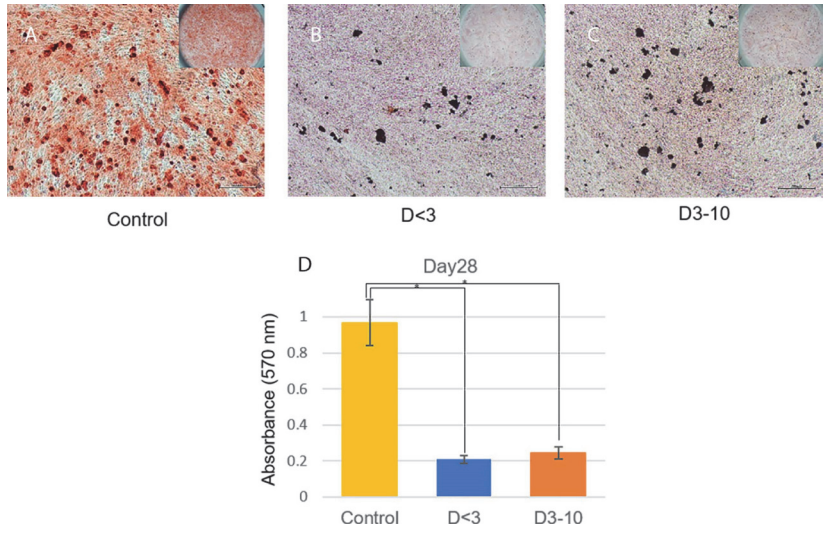

Figure 2 Alizarin red S staining at Day 28 results: Calcified nodule formation in A) Control group, B) D<3 group, and C) D3–10 group (bar scale = 200 μm), D) Comparison of the absorbance of the extracted alizarin red S staining of the three groups. *Statistically significant difference at $p<0.05$ (n=3)

SHED-CM was selected for our study due to its extensive range of regenerative benefits. Although previous studies used the entire SHED-CM to examine its biological effects, our research focused solely on the peptides in SHED-CM due to their small molecular weight, which would enhance their ability to either diffuse through or act as ligands that bind to specific receptors on the cell membrane. These peptides also have high target specificity, high affinity, and high potency of action [26]. Moreover, peptides rarely accumulate in specific organs, which minimizes the risk of toxicity [32] or adverse immune responses [33]. On the production level, the synthesis of peptides is also more cost-effective and time-efficient compared with other larger molecules [34].

To the best of our knowledge, this study is the first to reveal that SHED-CM fractions of less than 10 kDa, stimulates PDLSC proliferation, with the less than 3 kDa fraction being more effective in enhancing cell growth compared with the 3–10 kDa fraction. Other authors have demonstrated that SHED-CM enhanced the proliferation of other cell types, such as Schwann cells [23, 35] and pancreatic β-cells [24]. The secreted factors from the entire SHED-CM that were implicated in the upregulation of Schwann cell proliferation were the ectodomain of sialic acid-binding Ig-like lectin-9 (ED-Siglec-9) combined with monocyte chemoattractant protein-1 (MCP-1). In addition to these two factors, the neurotrophic factors in SHED-CM, such as NGF, BDNF, NT-3, CNTF and GDNF were also shown to promote Schwann cell proliferation. In the latter study, VEGF-A and PI3K in the SHED-CM was found to play an important role in pancreatic β-cell proliferation.

In addition to increased PDLSC proliferation, both SHED-CM fractions in our study inhibited PDLSC osteogenic differentiation. These findings are in contrast with previous studies that have demonstrated that SHED-CM stimulated osteogenic differentiation and promoted bone regeneration. Coating dental implant surfaces with SHED-CM has been associated with increased bone regeneration during the early stages of osseointegration [22]. Furthermore, Hiraki *et al* discovered that the SHED-CM fraction containing components larger than 10 kDa promoted bone regeneration and bone maturation in rat calvarial defects [21]. We hypothesize that these differences in results in the aforementioned studies could be due to their use of the entire SHED-CM that contains macromolecules, while our research used only the SHED-CM fraction containing components less than 3 kDa, which may comprise a different combination of factors.

The < 3kDa fraction used in our study was obtained by ultrafiltration followed by diethyl ether treatment, which is the most widely used solvent for peptide precipitation [36]. Compared with the D3–10 fraction, the D<3 fraction induced the most substantial increase in PDLSC proliferation. Further investigations are thus necessary to identify the peptides in SHED-CM that are responsible for the biological effects on the PDLSCs and to elicit the cellular mechanisms of these peptides to be able to apply this knowledge to future use.

There are several potential clinical approaches of SHED-CM, including enhancing bone repair for cleft patients and PDLSC proliferation or osteogenic differentiation to treat periodontal defects or accelerate tooth movement in orthodontic treatment. The CM can be used to implant scaffold [37] soaked with SHED-CM or injecting [38] SHED-CM into the defect area in the oral cavity. Future studies are required to apply this cell free-conditioned medium therapy in clinical situations. However, this study provides a greater understanding of the effect of SHED-CM on PDLSC proliferation and differentiation.

Conclusion

Currently, this is the first report to reveal that SHED-CM stimulated PDLSCs proliferation, with the most effective results seen from diethyl ether

precipitated <3 kDa SHED-CM (D <3). Precipitated SHED-CM at a concentration of 0.1 mg/ml, regardless of fraction size, suppressed calcified nodule formation on Day 28. The peptides from SHED-CM are potentially novel stimulators of PDLSC proliferation but may also inhibit osteogenic differentiation.

Conflicts of interest

The authors declare no conflicts of interest.

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