Original Article

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The proliferative effect of conditioned media from human periodontal ligament stem cells and human exfoliated deciduous teeth stem cells on human gingival fibroblasts

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Objective: This study aimed to investigate the potential of conditioned medium (CM) from human periodontal ligament stem cells (PDLSCs) and CM from stem cells from human exfoliated deciduous teeth (SHEDs) to promote the proliferation of human gingival fibroblasts (HGFs).

Materials and Methods: The PDLSC-CM, SHED-CM and HGF-CM were obtained from cell culture supernatants on day 2 and day 4. The amount of protein in each sample was determined using the Bradford assay. For the cell viability assay, HGFs were seeded in 96-well plates at a density of $1.2x10^4$ cells/well and treated with various concentrations of PDLSC-CM, SHED-CM and HGF-CM (8.75, 17.5, 35, 70, 140, or 280 µg/ml) in DMEM without serum for 24 hours. For the proliferation assay, HGFs were seeded in 96-well plates at a density of $2x10^3$ cells/well and treated with 8.75, 17.5, or 35 µg/ml PDLSC-CM, SHED-CM and HGF-CM in DMEM with serum. The proliferation of HGFs on days 1, 2, 4, 6, and 8 after treatment was measured.

Results: The cell viability of HGFs treated with PDLSC-CM at 8.75 µg/ml showed significantly greater cell viability than those treated with SHED-CM and HGF-CM at the same concentration. The proliferation assay showed that HGFs treated with a lower concentration of PDLSC-CM (8.75 µg/ml) had higher proliferation than cells treated with SHED-CM and HGF-CM at the same concentrations at day 4. Interestingly, HGFs treated with PDLSC-CM at 17.5 and 35 µg/ml exhibited higher proliferation than those treated with SHED-CM and HGF-CM for a longer period of time (4-6 days).

Conclusion: Our results suggest that PDLSC-CM enhances HGF proliferation more effectively than SHED-CM and that PDLSC-CM may be used as the promising agent for further studies on the biological properties of HGFs, which would be an advantage for oral and periodontal tissue healing and regeneration.

Keywords: cell proliferation, cell viability, conditioned medium, human gingival fibroblasts, periodontal ligament stem cells, stem cells from human exfoliated deciduous teeth

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Introduction

Periodontal disease is a chronic inflammatory condition involving a breakdown of the periodontium, periodontal pocket formation, and tooth loss [1]. The potential of the periodontal ligament (PDL) to regenerate from the loss of periodontal tissues is limited. The inability of existing treatment modalities such as bone grafting and guided tissue regeneration has shown unpredictable results. Thus, studies on the use of mesenchymal stem cells (MSCs) in periodontal tissue engineering and regeneration are increasing to achieve the complete periodontal regeneration [2]. MSCs are multipotent cells that play an important role in tissue homeostasis, repair, and regeneration [3]. The dental-derived MSCs such as periodontal ligament stem cells

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(PDLSCs), stem cells from human exfoliated deciduous teeth (SHEDs) and gingiva-derived MSCs (GMSCs) are easy to isolate from discarded tissues in routine dental clinics [4]. Moreover, both PDLSCs and SHEDs exhibit their high proliferation and osteogenic differentiation potential [5-6]. Therefore, PDLSCs and SHEDs may allow for periodontal regeneration using stem cell-based therapies.

PDLSCs have the ability to in vitro differentiate into multilineage cells including osteoblasts, chondroblasts, and adipocytes, and to generate cementum-like structures and dense collagen fibers that are similar to Sharpey's fibers in vivo [7]. SHEDs are found in the dental pulp of exfoliated deciduous teeth and have the ability to differentiate into neural cells, adipocytes, osteoblasts, and odontoblasts [8]. SHEDs transplanted in vivo using hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier enable to generate dentin/pulp-like structures, and show the osteo-inductive potential by recruiting host osteogenic cells [8]. Furthermore, the transplantation of SHEDs can repair calvarial bone defects in mice [9]. Although PDLSCs and SHEDs are able to undergo osteogenic differentiation [5], PDLSCs exhibit higher osteogenic potential than SHEDs, as demonstrated by higher alkaline phosphatase activity and histochemical staining in vitro [10, 11]. However, an in vivo study has reported that animals treated with HA/TCP combined with either allogenic PDLSCs or allogenic SHEDs show a similar ability to regenerate periodontal defects, as shown by the CT scan and histological examination [12].

While MSCs are a promising source for stem cell-based therapy, MSCs transplantation still has some limitations. For example, the cells exhibit poor differentiation and a poor survival rate, a limited number of cells reaches the target site, and there are safety concerns regarding immune compatibility and tumorigenicity [13]. Thus, cell-free based therapies using the mesenchymal stem cell derived conditioned media have gained increasing attention due to their therapeutic potential for regenerative medicine and have been proposed as a candidate for MSC-based therapy. Conditioned media (CM) are the media used for culturing stem cells for a period of time and containing a variety of secreted proteins, cytokines and chemicals. The stem cell-derived CM is free from immunogenicity. The CM also requires less sterile condition, compared to the direct stem cell transplantation. In addition, the CM is feasible to be manufactured and storage for extended periods with the stable properties. It can be prepared for proper dosages and produced in large quantities with a non-invasive, time and cost saving procedure [13].

Several studies have reported that the CM demonstrates positive effects on mesenchymal tissue regeneration. Linero *et al*. reported that CM from adipose-derived stem cells can repair rabbit mandibular defects in a similar manner as CM from bone marrow mesenchymal stem cells [14]. A study has previously reported that conditioned medium promotes rapid wound healing by increasing new blood vessel formation and collagen synthesis [15]. Zheng *et al*. studied the proliferative and differentiation potentials of aged PDLSCs upon treatment with CM from young PDLSCs. The results showed that aged PDLSCs could differentiate into cementum/PDL-like tissue. Thus, CM can enhance some biological properties for tissue regeneration [16]. Preclinical studies on animal models have shown that CM from dental stem cells have significant effects on tissue repair in various disease models, such as models of neurological diseases, joint/bone-related diseases, and periodontal defects [17]. Recently, Nagata *et al*. have analyzed micro-CT scans and histological sections from a rat model and reported that PDLSC-CM can enhance periodontal regeneration of defective periodontal tissue in a concentration-dependent manner [18]. Furthermore, they found that PDLSC-CM contains a mixture of proteins, including matrix proteins, enzymes, growth factors

such as insulin-like growth factor binding protein 6 (IGFBP6), IGFBP2, platelet-derived growth factor receptor β, cytokines, and angiogenic factors [18]. The effect of the various cooperative cytokines in PDLSC-CM, such as insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-β1), and hepatocyte growth factor (HGF), was suggested to induce periodontal regeneration [19].

Fibroblasts play an important role in wound healing of connective tissues and the periodontium. In the proliferative phase of wound healing, these cells migrate into the provisional matrix and produce extracellular matrix (ECM), e.g., collagen, proteoglycans, and fibronectin, which can support cell adhesion, proliferation, and differentiation [20]. Although human gingival fibroblasts (HGFs) are the most abundant cell type residing in gingival connective tissues and play an important role in oral and periodontal wound healing, HGFs exhibit a lower growth rate than PDL fibroblasts [21]. Furthermore, HGFs produce fewer ECM components, including collagen type I, III, and fibronectin [22, 23], and have a lower level of alkaline phosphatase [24] than PDL fibroblasts. Since HGF proliferation is limited, enhancing the regenerative potential of HGFs is challenging. Moreover, the potential of PDLSC-CM and SHED-CM to promote HGFs has not yet been reported. Thus, this study aimed to investigate the effects of PDLSC-CM and SHED-CM on the proliferation of HGFs. The results of this study may identify a potential strategy for improving HGF proliferation for applications for periodontal wound healing and periodontal regeneration in the future.

Materials and methods

Culture of PDLSCs and SHEDs

PDLSCs, purchased from ScienceCell Research Laboratories, CA, USA), were obtained from Dr. Sujiwan Seubbuk Sangkhamanee, Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University. SHEDs were obtained from Dr. Hathaithip Sritanaudomchai, Department of Oral biology, Faculty of Dentistry, Mahidol University. Both cell types were characterized their stem cell properties regarding Seubbuk *et al.* [25] and Goonmanee *et al.* [26], respectively. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution and incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every 2-3 days. Once reaching 80-90% confluency, the cells were sub-cultured as follows: the old medium was removed; the cells were washed twice with 1X of phosphate-buffered saline (PBS); 1 mL of 0.5% trypsin-EDTA was added; the cells were incubated for several minutes (min); detached cells were visualized under an inverted microscope; 9 mL new medium was added; the cell suspension was transferred to a centrifuge tube and centrifuged at 2,000 rpm for 5 min; the supernatant was removed; the cell pellet was resuspended in new medium before plated in a culture flask at a ratio of 1:3. The PDLSCs passage 7-9 and SHEDs passage 5 were used in the experiment.

Preparation of CM

After PDLSCs, SHEDs, and HGFs reached 80-90% confluency, CM was collected as follows: the old medium was removed, DMEM without FBS and antibiotic-antimycotic solution was added, and the cells were incubated for another 2 days. The cell culture supernatants were collected from days 2 to 4. Then, the CM was centrifuged at 6,000 rpm and 4°C for 5 min, and the supernatant was filtered through a 0.2 μm sterile syringe filter. The CM was stored at -20°C until further use.

Determination of protein concentration

The Bradford assay was used to measure the concentration of total protein in each sample.

The 195 µl of Bradford reagent was added to each well of a 96-well plate. Then, 5 µl of 0.125, 0.25, 0.5, and 0.75 mg/ml bovine serum albumin (BSA) and CM were added. The plates were then incubated for 15 min, and the absorbance value of each well was measured using a microplate reader at 595 nanometers (nm). The total protein concentration in each sample was estimated by comparison to protein standards.

Culture of HGFs

The HGF cell line (ATCC CRL-2014™) was obtained from Assoc. Prof. Dr. Rudee Surarit, Department of Oral Biology, Faculty of Dentistry, Mahidol University. The cells were cultured in DMEM comprising 10% FBS, 100 units/mL penicillin, and 100 µg/ml streptomycin and incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every 2-3 days. When cells reached 80-90% confluency, they were subcultured as described above. HGFs from passages 7-9 were used for the experiments. Trypan blue stain (0.4%) was added to the HGFs, and the samples were placed in a hemocytometer to count viable cells.

Cell viability assay

HGFs were seeded in 96-well plates at a density of $1.2x10^4$ cells/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours (h). Cells were cultured with DMEM without FBS and treated with different concentrations (8.75, 17.5, 35, 70, 140, or 280 µg/ml) of the following types of medium: a) PDLSC-CM, b) SHED-CM, c) HGF-CM, or d) DMEM without FBS as a negative control. Then, cells were incubated for 24 h. After that, the cells were washed twice with PBS, and 100 µl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well. The cells were incubated for 2 h. Then, the MTT solution was removed, and the cells were washed with PBS. The purple formazan crystals were dissolved by the addition of 100 µl dimethyl sulfoxide (DMSO). The absorbance of the formazan in each well was measured using a microplate reader at 570 nm and reported as the optical density (OD). The procedure was carried out in triplicate. The percent cell viability was calculated based on the following formula. Percent cell viability = OD (sample) - OD (blank) /OD (control) - OD (blank) x100. This assay was independently repeated three times and each group was run triplicates per experiment.

Cell proliferation assay

HGFs were seeded in 96-well plates at a density of $2x10^3$ cells/well and incubated at 37°C in a 5% CO_2 atmosphere. The cells were cultured with DMEM comprising 10% FBS and treated with different concentrations (8.75, 17.5, or 35 µg/ml) of the following types of medium: a) PDLSC-CM, b) SHED-CM, c) HGF-CM, or d) DMEM comprising 10% FBS as a negative control. Cell proliferation was measured by the MTT assay on days 1, 2, 4, 6 and 8 after treatment as described above. The absorbance of formazan in each well was measured using a microplate reader at 570 nm and the reported as the optical density (OD). To estimate the number of cells, a standard curve of the ODs of known cell numbers (10^3, $5x10^3$, 10^4, $3x10^4$, and $5x10^4$ cells) for the MTT assay was established. This assay was independently repeated three times and each group was run triplicates per experiment.

Statistical analysis

The data from the experiments are presented as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess the normality of the data. The data were analyzed using one-way ANOVA followed by Tukey's HSD. Differences were considered as a statistical significance when $p<0.05$.

Results

Determination of protein concentration

The Bradford assay was used to measure the total protein concentration of all samples. The total protein concentration of each sample was estimated by a comparison to protein standards (Suppl. Figure 1). The estimated protein concentrations of cells treated with PDLSC-CM, SHED-CM, and HGF-CM were 461, 554, and 281 µg/ml, respectively (Suppl. Table 1). Then, the effect of 2-fold dilutions of PDLSC-CM, SHED-CM, and HGF-CM at a concentration of 280 µg/ml on the viability of HGFs was evaluated.

Morphology of HGFs

HGFs were cultured in DMEM without 10% FBS at 37°C in a 5% CO_2 atmosphere. Cells were treated with SHED-CM or PDLSC-CM or HGF-CM at 280 µg/ml, the highest concentration used in this study, for 24 h (Figure 1). The results showed that the morphology of the control cells (Figure 1A), SHED-CM-treated cells (Figure 1B), PDLSC-CM-treated cells (Figure 1C) and HGF-CM treated cells (Figure 1D) exhibited spindle-shaped. The morphology of HGFs treated with SHED-CM or PDLSC-CM or HGF-CM was unchanged.

Cell viability assay

To assess the viability of HGFs, cells were treated with PDLSC-CM, SHED-CM, or HGF-CM at a concentration of 8.75, 17.5, 35, 70, 140, or 280 µg/ml for 24 h. The percent cell viability of HGFs was 115.38, 116.55, 117.77, 114.98, 108.05, and 97.42 upon treatment with SHED-CM at concentrations of 8.75, 17.5, 35, 70, 140, and 280 µg/ml, respectively. The results demonstrated that there was a significant increase in percent cell viability ($p<0.05$) upon treatment with SHED-CM at concentrations ranging from 8.75-70 µg/ml, compared to the untreated cells (the percent cell viability above than 100) (Figure 2).

The percent cell viability of HGFs was 138.11, 132.90, 128.88, 115.14, 103.48, and 100.16 upon treatment with PDLSC-CM at concentrations of 8.75, 17.5, 35, 70, 140, and 280 µg/ml, respectively. The results demonstrated that there was a significant increase in percent cell viability ($p<0.05$) upon treatment with PDLSC-CM at concentrations lower than 70 µg/ml, compared to the untreated cells (the percent cell viability above than 100) (Figure 2).

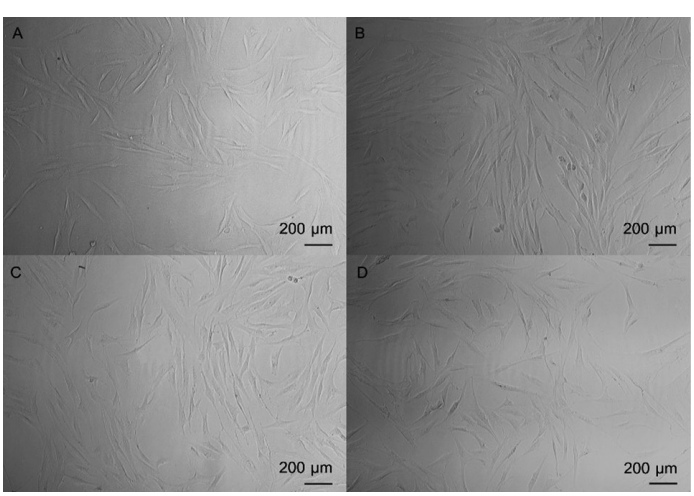


Figure 1 The morphology of HGFs after cultured in DMEM without FBS (control) (A), SHED-CM (B), PDLSC-CM (C), and HGF-CM (D) at 37°C in 5% CO_2 atmosphere for 24 h. Scale bar = 200 µm.

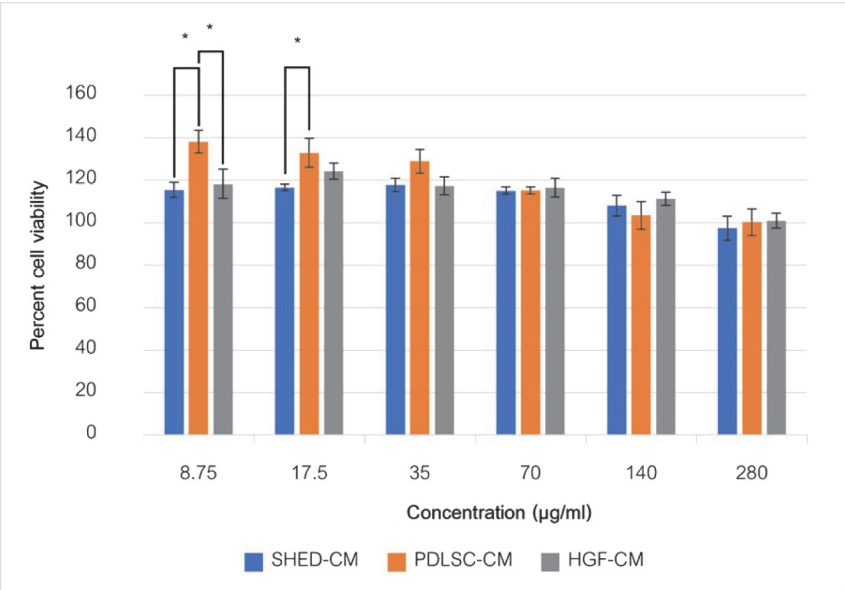


Figure 2 Percent cell viability of HGFs treated with SHED-CM, PDLSC-CM, and HGF-CM at the concentrations of 8.75, 17.5, 35, 70, 140, and 280 µg/ml, respectively (mean ± SD, n = 3). A significant difference is shown with an asterisk (*) at $p<0.05$.

The percent cell viability of HGFs was 118.26, 124.22, 117.34, 116.44, 111.27, and 100.86 upon treatment with HGF-CM at concentrations of 8.75, 17.5, 35, 70, 140, and 280 µg/ml, respectively. The results demonstrated that there was a significant increase in percent cell viability ($p<0.05$) upon treatment with HGF-CM at concentrations lower than 70 µg/ml, compared to the untreated cells (the percent cell viability above than 100) (Figure 2).

The effects of SHED-CM, PDLSC-CM, and HGF-CM at the same concentration were compared (Figure 2). There were no statistically significant differences in percent cell viability between cells treated with SHED-CM and HGF-CM at any concentration. Interestingly, HGFs treated with PDLSC-CM had a significantly higher percent viability ($p<0.05$) than those treated with HGF-CM at a concentration of 8.75 µg/ml. Moreover, HGFs treated with PDLSC-CM had a higher percent viability than those treated with SHED-CM at concentrations of 8.75 and 17.5 µg/ml.

Proliferation assay

Based on the cell viability results, SHED-CM, PDLSC-CM, and HGF-CM at concentrations of 8.75, 17.5, and 35 µg/ml significantly increased HGF viability. Thus, CM at these concentrations were used for the proliferation assay. To estimate the number of cells, the OD at 570 nm was plotted against the number of cells (Suppl. Figure 2).

On day 4, the results demonstrated that PDLSC-CM and SHED-CM promoted higher cell proliferation than the control. On day 6, the proliferation of cells treated with PDLSC-CM at concentrations of 8.75, 17.5 and 35 µg/ml was higher than that of control. In contrast, SHED-CM slightly reduced cell proliferation at concentrations of 8.75, 17.5 and 35 µg/ml (Figure 3).

The effects of SHED-CM, PDLSC-CM, and HGF-CM at the same concentration were compared. The results showed that PDLSC-CM promoted higher cell proliferation than SHED-CM at all concentrations on day 4 and day 6. On day 6, both PDLSC-CM and SHED-CM at a concentration of 35 µg/mL induced the highest cell proliferation.

Consequently, all types of CM were used at a concentration of 35 µg/ml for a comparison. The results showed that 35 µg/ml PDLSC-CM induced significantly higher cell proliferation than 35 µg/ml SHED-CM on days 4-6 (Figure 3).

Discussion

Periodontal damage can cause an inadequate masticatory function in patients [27]. Although a stem cell-based therapy is a potential therapeutic approach in periodontal tissue regeneration, some limitations have recently reported such as a poor survival rate of transplanted cells and immunogenicity, etc. Nowadays, stem cell-free therapy has gained more attention by using the bioactive factors secreted from stem cells for the regenerative purposes [13]. Using the conditioned media or CM which is a biological waste normally discarded during the laboratory procedure is currently an attractive strategy for the stem cell-free therapy. Although MSCs can be isolated from various dental tissues, this study focuses on PDLSCs and SHEDs due to their high proliferative and osteogenic properties. Both cells have been previously reported their MSC markers, ability to form the colony-forming unit and in vitro multi-differentiation, indicating their stem cell properties [25, 26].

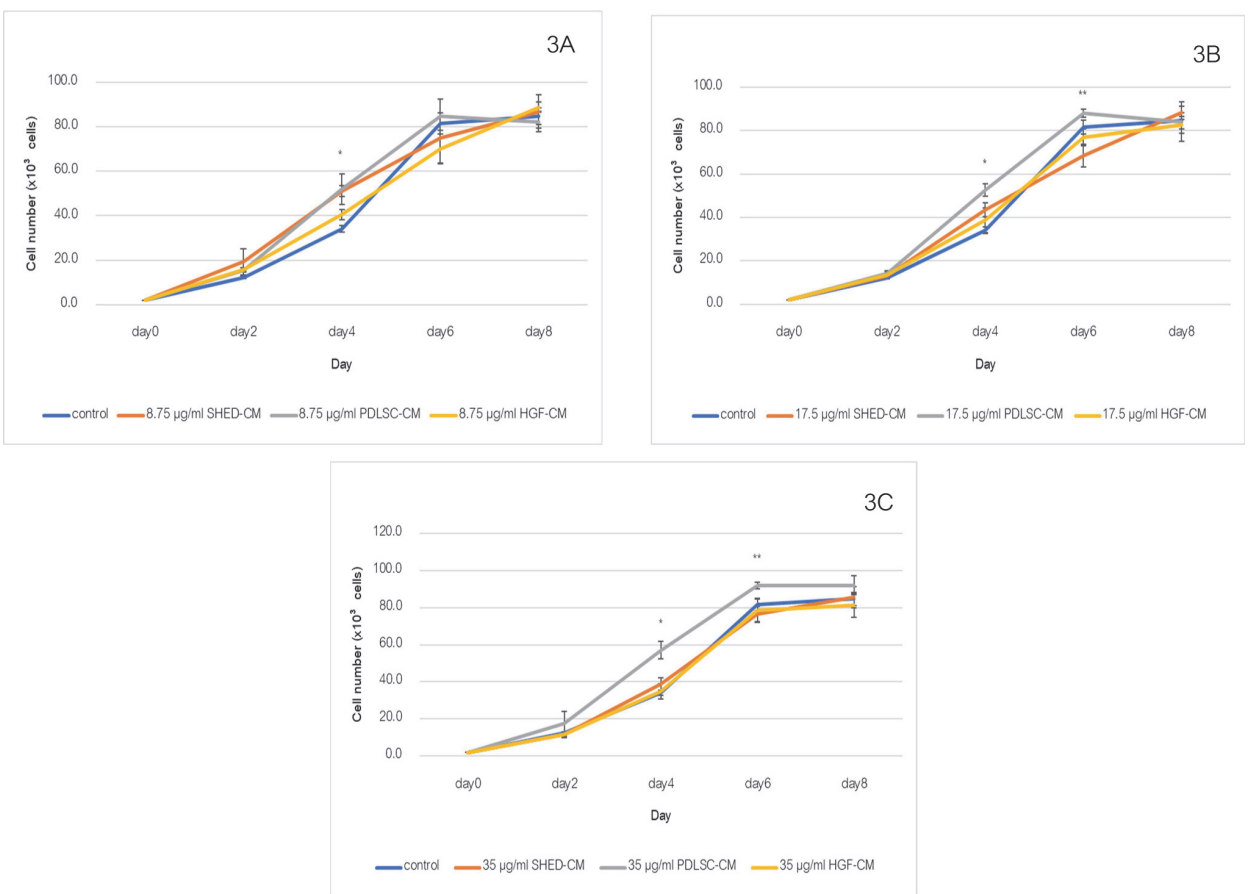


Figure 3 Cell proliferation assay of HGFs treated with SHED-CM, PDLSC-CM, and HGF-CM at a concentration of 8.75 µg/ml (3A), 17.5 µg/ml (3B), and 35 µg/ml (3C). (*) indicates a significant difference in cell number of PDLSC-CM, compared to SHED-CM, HGF-CM, and control. (**) indicates a significant difference in cell number of PDLSC-CM, compared to SHED-CM and HGF-CM. $p<0.05$.

The methods used for isolation and storage of CM is an important consideration for bioprocessing of MSC products because these methods may affect the variability of MSC products. For instance, the type and number of products secreted from MSCs may vary between batches depending on the culture period or culture growth phase [28]. To minimize the effect of the variation in the amounts of proteins secreted from MSCs, the Bradford assay was used to examine the protein concentration and the protein concentration in each batch of PDLSC-CM, SHED-CM and HGF-CM was adjusted into the estimated same concentration.

Cell viability and proliferation are primarily considered as essential criteria to determine the cell biological property. Cell viability is the quantification of the number of living cells at each time point whereas cell proliferation is described as the measurement of dividing cells or as the cellular growth rate among various time points. Prior to the proliferation assay, the cell viability was conducted to rule out the non-toxic concentrations of CM from PDLSCs, SHEDs and HGFs to the cells. Since the doubling time of HGFs is approximately 18-24 h [29], the cell viability was investigated at 24 h. Next, the proliferation of HGFs was performed to cover the period of doubling time at day 2, 4, 6 and 8.

Overall, the cell viability assay demonstrated that PDLSC-CM, SHED-CM and HGF-CM at concentrations lower than 280 µg/ml were not toxic to HGFs. Additionally, CM at concentrations of 8.75, 17.5 and 35 µg/ml significantly increased the viability of HGFs. According to the cell viability, HGFs treated with 280 µg/ml PDLSC-CM, SHED-CM, and HGF-CM showed lower cell viability than those treated with these types of CM at lower concentrations, possibly due to the presence of metabolic waste from MSCs. A previous study has shown that byproducts such as ammonium and lactate could inhibit the cell proliferation [30]. However, the normal cell morphology of HGFs among different groups of CM at 280 µg/ml was still observed, compared with that of the untreated cells (control group). Regarding the cell viability, the optimal concentrations of CM for culturing HGFs which were 8.75, 17.5 and 35 µg/ml were used in the proliferation assay.

The cell proliferation assay showed that PDLSC-CM more strongly promoted HGF proliferation than SHED-CM at concentrations of 8.75, 17.5 and 35 µg/ml on days 4-6. This may be explained by the different components of the two CMs. PDLSC-CM contains a mixture of proteins, including matrix proteins (type I collagen, fibronectin, etc.), enzymes, growth factors (IGFBP6, IGFBP2, platelet-derived growth factor-AA, etc.), cytokines, and angiogenic factors (VEGF, urokinase-type plasminogen activator, etc.) [19]. On the other hand, SHED-CM contains IGFBP-6, tissue inhibitor of metalloproteinase 2 (TIMP-2), TIMP-1, TGF-β1, IGFBP-2, IGFBP-4, BMP-5, and other factors [31]. The components of PDLSC-CM may be responsible for efficiently promoting HGF proliferation.

In addition to various components described above, certain current studies demonstrate the presence of secretomes in the stem cell-derived conditioned media. The secretome, which is secreted by MSCs into the extracellular environment, contains bioactive molecules acting on the same cell (autocrine signaling) or nearby cells (paracrine signaling). The secretome consists of cytokines, growth factors, free nucleic acids, lipids, and extracellular vesicles (EVs) [32, 33]. Numerous studies have shown that the therapeutic effects of the MSC secretome are similar to those of transplanted MSCs in various disease models in vivo [34]. A previous study has demonstrated that stem cells derived from different anatomic locations show a variation in the secretome profile [32]. This suggests that a difference in secretomes in PDLSC-CM and SHED-CM may influence a dissimilar response of HGFs. Nevertheless, various factors and secretomes are needed to be further identified.

On days 4 and 6, PDLSC-CM enhanced HGF proliferation in a dose-dependent manner and 35 µg/ml was the optimal concentration. This is consistent with several studies reporting the positive effect of various types of MSC-CM. For instance, Li *et al*. demonstrated that umbilical cord-condition medium (UC-MSC-CM) increases the proliferation of dermal fibroblasts and suggested that UC-MSC-CM promotes cutaneous wound healing with few scars [35]. Additionally, Inukai *et al*. demonstrated that human bone marrow-derived MSC-CM promotes the migration and proliferation of canine PDL cells [19].

In periodontal wound healing, one of the important steps for favorable healing is the attachment of collagen fibers in the lamina propria of the gingiva to the root surface and alveolar bone, indicating the importance of matrix-producing cells [36]. Soluble mediators such as cytokines, chemokines and growth factors induce the proliferation, migration and collagen synthesis of fibroblasts [37]. Our study demonstrated that PDLSC-CM enhanced the proliferation of HGFs; nonetheless, further studies are needed to investigate the effect of PDLSC-CM on the biological properties of HGFs which are important for tissue healing or regeneration such as cell migration and collagen synthesis, etc.

Studies in animal models have shown the potential of stem cell transplantation, including PDLSC transplantation, to regenerate defective periodontal tissue [38]. However, it has been revealed that transplanted PDLSCs do not survive in the microenvironment of the recipient site. Instead, a possible strategy for promoting wound healing in the periodontium is the use of paracrine factors released from PDLSCs [39]. Nagata *et al*. found that PDLSC-CM enhances periodontal tissue regeneration in a rat periodontal defect model [40]. Thus, the application of products that involve PDLSC-CM, such as scaffolds combined with PDLSC-CM, may improve periodontal tissue regeneration via cell-free therapies.

By physiologic location, gingival fibroblasts in gingiva are just next to the PDL cells in the PDL space. The secretion from PDL cells could diffuse to nearby area and enhance the HGF properties in the normal circumstance. However, in certain niches such as aging or underlying systemic conditions, endogenous cells and environments might have an inefficient regenerative potential. With our hope, using the PDLSC-derived CM which contains a variety of bioactive growth factors may improve the ability of those oral cells to regenerate and function properly.

In conclusion, this study suggests that PDLSC-CM enhances HGF proliferation more strongly than SHED-CM and HGF-CM. The significant increasing of cell viability and proliferation among the treatment with PDLSC-CM, SHED-CM and HGF-CM implies a different capacity among different cell types to secrete potential molecules affecting HGFs. Future investigations are also needed to examine the effect of PDLSC-CM on the migration and collagen synthesis of HGFs. The results of this study may lead to the development of an effective product for improving periodontal wound healing or periodontal tissue regeneration.

Acknowledgments

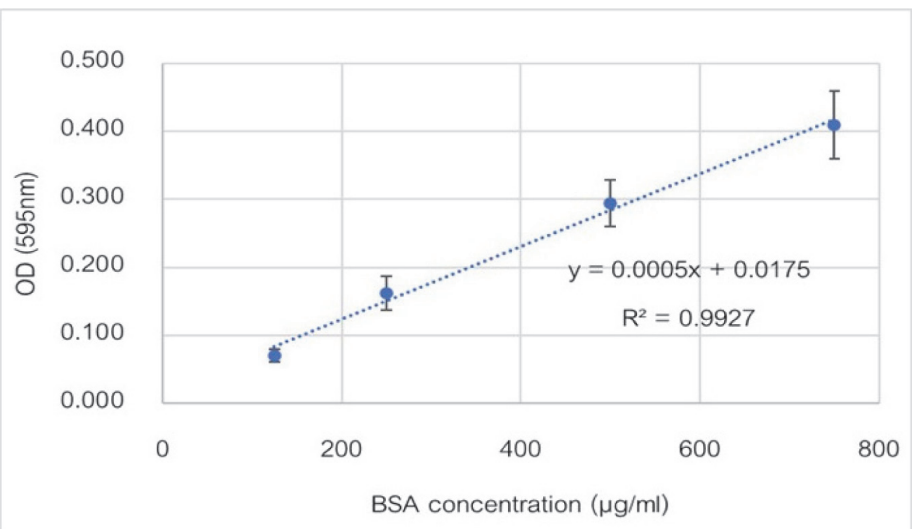
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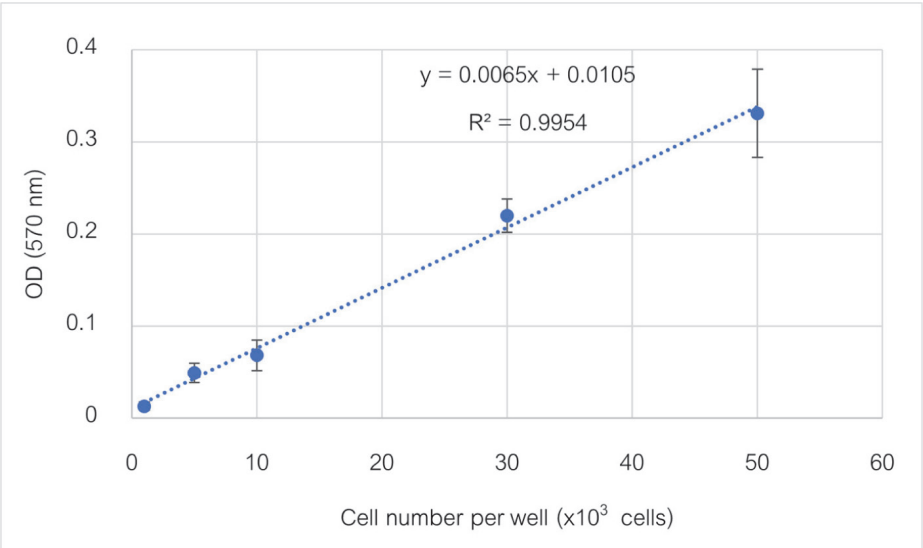
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Supplementary Figure 1 Bovine serum albumin (BSA) standard curve. The estimated protein concentration was calculated using the equation: y = 0.0005x + 0.0175, where y= OD (Optical Density) and x is the BSA concentration in µg/ml.



Supplementary Figure 2 The MTT standard curve. The estimated cell number per well was calculated using y=0.0065x + 0.10105, where y= OD and x= cell number per well (x10^3 cells).

Supplementary Table 1 The estimated protein concentration (μg/ml) of PDLSC-CM, SHED-CM, and HGF-CM using Bradford assay.

Sample	OD	Estimated protein concentration (μg/ml)
PDLSC-CM	0.263	461
SHED-CM	0.313	554
HGF-CM	0.167	281