Tissue and Cell 65 (2020) 101373

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

A compound of concentrated growth factor and periodontal ligament stem cell-derived conditioned medium

Z. Aghamohamadi[a,b], M. Kadkhodazadeh[c], M. Torshabi[b], F. Tabatabaei[a,b,d,*]

[a] Dental Research Center, Research Institute of Dental Sciences, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Dental Biomaterials, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Periodontics Department, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Marquette University School of Dentistry, Milwaukee, WI, USA

ARTICLE INFO

Keywords:
Concentrated growth factor
Conditioned media
Periodontal ligament stem cells
Secretome
Tissue engineering

ABSTRACT

The aim of this study was to determine the *in vitro* effect of a compound of concentrated growth factor (CGF) and periodontal ligament stem cell-derived conditioned medium (PDLSCs-CM) as a potential product for future applications in periodontal tissue regeneration. Isolated PDLSCs were characterized using flow cytometry and differentiation into osteoblasts and adipocytes cells. PDLSCs-CM and CGF were prepared and lyophilized. To determine the optimal concentration of the CGF-CM compound, the proliferation of PDLSCs after exposure to a wide range of different concentrations of CGF, CM, or their combination (CGF + CM) was investigated by methyl thiazol tetrazolium assay. Successful isolation of PDLSCs was confirmed by high expression of mesenchymal surface markers and differentiation into osteoblasts and adipocytes. PDLSCs showed higher proliferation in the 6.25 mg/mL concentration of CM and 94 μg/mL concentration of CGF. High concentrations of CGF and CM markedly inhibited the proliferation of PDLCs ($p < 0.05$). The exposure of PDLSCs to the compound of 10% CM + 90% CGF significantly increased the cell proliferation ($p < 0.05$). The results showed that CGF, CM, or their combination exert a proliferative effect on cells at a certain concentration. Further investigation on the synergistic effect of this compound may approve its application for periodontal regeneration.

1. Introduction

Periodontal diseases are recognized as the second infectious oral diseases which can cause the tooth loss. The purpose of periodontal treatment is to restore the physiological function of the teeth supportive tissues, including the alveolar bone, gingiva, periodontal ligament (PDL), and root cementum. Conventional clinical treatment strategies like scaling, root planing, and guided tissue/bone regeneration have failed to completely reconstruct periodontal tissue. Regenerative dentistry is rapidly developing towards non-invasive treatments to achieve a more predictable and desirable tissue regeneration. The main components of tissue engineering approach which are stem cells, growth factors, and scaffolds can be used for regeneration of damaged periodontal tissues (Carmagnola et al., 2019; Fretwurst et al., 2018).

Stem cells with self-renewal and multi-lineage differentiation properties have a high potential for disease treatment. At the same time, one of the major drawbacks of cell therapy is the need for a large number of cell (Xu et al., 2019). Bone marrow mesenchymal stem cells (BMMSCs) are the first discovered stem cells, which have the capability of improving periodontal defects (Kawaguchi et al., 2004). Oral and dental tissues also contain a variety of stem cells. Among these cells, periodontal ligament stem cells (PDLSCs) and dental follicle cells have been widely used for periodontal regeneration (Zhu and Liang, 2015). The high cost of stem cell separation, the likelihood of moving cells from damaged tissue, as well as the possibility of tumorigenesis, encouraged researchers to look for the mechanism of action of stem cells. The stem cells' secretory molecules - referred to as secretome, microvesicles, or exosome - exert paracrine actions on host tissues and have an important role in stem cell-mediated reparative effects. These factors, which can be found in the medium of cultured stem cells, comprises a diverse array of serum proteins, growth factors, angiogenic factors, hormones, cytokines, extracellular matrix proteins, extracellular matrix proteases, and hormones (Muhammad et al., 2018). Lack of cells in the secretome containing conditioned medium (CM), the possibility of freeze-drying, more easily transporting than cell-containing materials, and no requirement for donor and recipient matching are several advantages of CMs over direct application of stem cells (Pawitan, 2014).

* Corresponding author at: Marquette University School of Dentistry, Milwaukee, WI, USA.
E-mail address: fahimeh.tabatabaei@marquette.edu (F. Tabatabaei).

https://doi.org/10.1016/j.tice.2020.101373
Received 20 April 2020; Accepted 5 May 2020
Available online 13 May 2020
0040-8166/ Published by Elsevier Ltd.

In tissue engineering, growth factors are required for initiating stem cell activity. Growth factors can be delivered to the cell in various ways (Ren et al., 2019). In recent years, the application of platelet-derived bio-products like platelet-rich-plasma (PRP), plasma-rich in growth factors (PRGF), or platelet-rich fibrin (PRF) from the patient's own blood in periodontal regeneration has received much attention. Alpha-granules in platelets contain various growth factors which can be released by platelets activation and stimulate tissue regeneration (Acebes-Huerta et al., 2019). Concentrated growth factor (CGF) is the latest introduced form of platelet derivative that contains thick fibrin matrix and a high amount of growth factors and cytokines. This product is used as fresh, freeze-dried, exudate, or membrane in various studies to improve tissue repair (Tabatabaei et al., 2020).

Using the proper combination of stem cells and growth factors can play an important role in tissue regeneration. Since the combination of CGF and PDLSCs-CM has not been studied so far and considering the presence of growth factors in both products, we hypothesized a possible synergistic action between them. Our study aimed to establish an optimal combination of PDLSCs-CM and CGF for future applications in periodontal tissue regeneration.

2. Materials and methods

2.1. Ethical aspects

This work was conducted following The Code of Ethics of the World Medical Association (Declaration of Helsinki), and by obtaining the informed consent from human subjects. The study is approved by the Ethics Committeeof Shahid Beheshti University of Medical Sciences (# IR.SBMU.RIDS.REC.1395.325).

2.2. Isolation and characterization of hPDLSCs

Periodontal ligament stem cells (PDLSCs) were isolated from the healthy periodontal ligament tissue of permanent premolar teeth of young individuals. Teeth had no sign of caries, periapical lesion, gingivitis, and/or periodontal disease and were to be extracted for orthodontic purposes. Before extraction, the teeth were polished, and the patients were asked to use chlorhexidine mouthwash for one minute. After performing local anesthesia, conservative extraction with minimal wobbling was done. The periodontal ligament tissue was separated from the middle area of the tooth root, and the stem cells were isolated using the explant culture. Briefly, the periodontal tissue was minced into small pieces and incubated in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA), supplemented with 15% fetal bovine serum (FBS; Gibco), and 1% of antibiotic-antimycotic (Biosera, USA) at 37 ˚C in a humid atmosphere with 5% CO_2. Third passage cells were used for all experiments.

Surface expression of MSC-associated markers of cells isolated from periodontal tissue was analyzed by flow cytometry. For flow cytometric analysis, suspension of cells (1 × 107 cell/mL) with fluorescein isothiocyanate (FITC) conjugated anti-human CD90 (Exbio, Vestec, Czech Republic), anti-CD105-FITC (Exbio), anti-CD73-FITC (BD Biosciences, San Jose, CA), anti-CD45-FITC (Exbio), and Phycoerythrin (PE) anti-human CD34 (Exbio) were incubated for 1 h at 4 ˚C, washed with PBS, and analyzed using BD FACSCalibur system (BD Biosciences, San Jose, CA, USA).

To evaluate the plasticity of isolated cells, cells were seeded in 6-well plates (25,000 cell/well and 50,000 cell/well for adipogenic and osteogenic differentiation, respectively). After reaching subconfluent stage, the media was replaced with osteogenic (DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic, 10 nM dexamethasone, 200 μM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate) or adipogenic (DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic, 100 μg/mL isobutyl-methylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin) induction media for 14, 21 days respectively.

Table 1
Primers used for evaluating the expression of the studied genes.

Gene name	Sequence (5′ - > 3′)	Product Length (bp)
OSX	F: GCCAGAAGCTGTGAAACCTC R: TGATGGGGTCATGGTGTCTA	121
OCT-4	F: TCGAGAACCGAGTGAGAGGC R: CACACTCGGACCACATCCTTC	121
ALP	F: ATTTCTCTTGGGCAGGCAGAGAGT R: ATCCAGAATGTTCCACGGAGGCTT	118
OP	F: AGAATGCTGTGTCCTCTGAAG R: GTTCGAGTCAATGGAGTCCTG	146
OC	F: CAGCGAGGTAGTGAAGAGAC R: TGAAAGCCGATGTGGTCAG	144
RUNX2	F: TCTTAGAACAAATTCTGCCCTTT R: TCTTAGAACAAATTCTGCCCTTT	136
COL1	F: TGTGGCCCAGAAGAACTGGTACAT R: ACTGGAATCCATCGGTCATGCTCT	89
CD44	F: CTGCCGCTTTGCAGGTGTA R: CATTGTGGGCAAGGTGCTATT	109
CD90	F: ATCGCTCTCCTGCTAACAGTC R: CTCGTACTGGATGGGTGAACT	135
GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	197

OSX: Osterix; Oct-4: octamer-binding transcription factor 4; ALP: Alkaline Phosphatase; OP: osteopontin; OC: osteocalcin; RUNX2: Runt-related transcription factor 2; COL1: type I collagen; CD44: Cluster of Differentiation 44; CD90: Cluster of Differentiation 90; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Alizarin Red S staining (2% alizarin with pH = 4.4 − 2.4 for 30 min at 37 ˚C) (Sigma) and Oil Red O staining (0.3% for 15 min) (Sigma) were used for evaluating osteogenic and adipogenic differentiation.

Changes in gene expression after osteogenic differentiation were also evaluated with quantitative reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted using the RNeasy Mini Plus Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. The purity and concentration of the extracted RNA were determined using a nano-drop spectrophotometer (mySPEC, Vienna, Austria) at 260 and 280 nm wavelengths. Then, cDNAs were synthesized from extracted RNAs using the QuantiTect® Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and amplified using the SYBR®Green PCR Master Mix (Qiagen, Hilden, Germany) on the ABI StepOne™ detection system (Applied Biosystems, CA, USA). The sequences of the specific primers (Bioneer, Korea) used in this study are shown in Table 1. The gene expression of GAPDH was used as a reference in all applications.

2.3. Preparation of conditioned medium (CM)

The CM was prepared according to previous studies with minor modifications (Al-Sharabi et al., 2017; Paschalidis et al., 2014; Yamaguchi et al., 2015). Third passage PDLSCs were seeded in cell culture flasks (75 cm^2) in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic-antimycotic (Gibco). To generate CM, at 70–80% confluence, the cells were washed with phosphate-buff ;ered saline (PBS; Gibco), the media were replaced with DMEM containing 0.5% FBS, and cells were incubated at 37 ˚C and 5% CO_2. After 48 h, the media were collected, centrifuged two times at 3000 g (3 min) and 1500 g (5 min), filtered (0.2 μm), and stored at −20 ˚C. Finally, the collected supernatants were lyophilized and stored at 4 ˚C until use.

2.4. The optimal concentration of CM

In order to investigate the effect of different concentrations, the maximum amount of CM powder that did not precipitate after 10 min was dissolved in DMEM. The pH of the sample was measured by pH-meter (Jenway, England) to ensure its neutrality, then serial dilution was performed.

Table 2
The experimental groups of cells exposed to different concentrations of CM.

J	I	H	G	F	E	D	C	B	A	Group
mg/mL	mg/mL	mg/mL	mg/mL	g/mL	g/mL	g/mL	g/mL	g/mL	g/mL	Unit
1.5	3	6.25	12.5	0.025	0.05	0.1	02	0.4	0.8	Concentration
T	**S**	**R**	**Q**	**P**	**O**	**N**	**M**	**L**	**K**	**Group**
µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	Unit
1.5	3	6	11.75	23.5	47	94	187	375	750	Concentration

The viability of PDLSCs exposed to different concentrations of CM was assayed using the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Steinheim, Germany) assay. Briefly, cells were seeded onto 96-well plates at a cell density of 3000 cells/well in regular medium for 24 h, then the media was replaced with DMEM containing 0.5% FBS. The next day, cells were treated with serially diluted CM (0.8 g/mL to 1.5 µg/mL) for 48 h. Then, the culture medium was replaced with MTT solution (0.5 mg/mL in serum-free culture medium) and after 3 h incubation, the resulting formazan crystals in cells was dissolved by dimethyl sulfoxide (Sigma-Aldrich, Steinheim, Germany). Discoloration absorbance was analyzed at a wavelength of 570 nm with 620 nm filter using a microplate reader (Anthos 2020, Salzburg, Austria). Percentage viability was defined as the relative absorbance of all experimental groups. Table 2 shows the experimental groups exposed to different concentrations of CM.

2.5. Preparation of concentrated growth factor (CGF)

Isolation of CGF was performed as proposed by Rodella et al. (2011). Briefly, venous blood was collected from healthy volunteers (25–45 years old) in sterile Vacuette tube without anticoagulant additives. The collected blood samples were immediately placed in a special centrifuge (Medifuge; Silfradentsrl, Sofia, Italy) and processed automatically according to the manufacturer's instructions (30-s acceleration, 2 min at 408 g, 4 min at 323 g, 4 min at 408 g, 3 min at 503 g, and 36-s deceleration and stop). At the end of the process, the CGF was formed in the middle layer of a three-layered product. The isolated CGF was stored at -20 °C for 24 h, then lyophilized in a freeze dryer (Pishgam, Iran).

2.6. The optimal concentration of CGF

The maximum amount of freeze-dried CGF that can dissolve in DMEM without precipitation was determined as 100 mg/mL. The solution was filtered (0.2 µm) and serially diluted with DMEM until 750 ng/mL (Table 3). The PDLSCs were plated into 96-well plates (3000 cells/well) in DMEM containing 10% FBS. After 24 h, the medium was replaced with DMEM containing 0.5% FBS. The next day, cells were exposed to different concentrations of CGF, 10% (positive control) or 0.5% (negative control) FBS. After 48 h of incubation, MTT assay was performed as described earlier.

2.7. Effect of CGF-CM on PDLSCs proliferation

After a comprehensive analysis of the results of MTT assays on CGF and CM in previous steps, the concentrations of 6.25 mg/mL CM and 94 µg/mL CGF were selected for preparing mixtures of CGF-CM. These concentrations were defined as 100%. The experimental groups were built as a mixture of 90%CGF + 10% CM to 10% CGF + 90% CM and were evaluated by MTT assay. Positive and negative control groups were cultured in medium containing 10% and 0.5% FBS, respectively. The optimal concentration of CGF-CM in this step was used for the next steps.

In order to evaluate the effect of time on cell proliferation in the presence of the optimal concentrations of CGF-CM, PDLSCs were seeded in 96-well plates as mentioned before and incubated for 24 h. After replacing the medium with DMEM containing 0.5% FBS and incubation for 24 h, cells were treated with CGF-CM, 10% (positive control) or 0.5% (negative control) FBS. Cell proliferation was assessed after 24 and 72 h of incubation by MTT assay.

2.8. Statistical analysis

The one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test was used for data analysis. $p < 0.05$ was considered to indicate a statistically significant difference. The analysis was performed using the Graph Pad Prism 8.0 software.

3. Results

3.1. Isolation and characterization of PDLSCs

The outgrown PDLSCs from the explant exhibited a spindle appearance similar to those of mesenchymal stem cells. Immunofluorescent analysis revealed positive expression of CD90 (99.7%), CD105 (99.9%), CD73 (99.1%), and negative expression of CD45 and CD34 (< 0.5%) (Fig. 1).

The adipogenic differentiation of stem cells was confirmed by Oil red O staining that showed the formation and accumulation of vacuoles containing lipids inside the cells. The formation of calcified nodules inside cells, seen with Alizarin Red staining, demonstrates the osteogenic differentiation ability of isolated cells (Fig. 2b and c). Control groups grown in the regular medium were negative for Oil red O and Alizarin Red staining (Fig. 2a). As shown in Fig. 2d based on the quantitative RT-PCR analyses, the expression of OCT4 (stem cells marker) and Runx2 (primary marker of osteogenic differentiation) genes in cells cultured in the osteogenic medium did not differ significantly from the control group in the regular medium (p-value > 0.05); however, down-regulation of CD44 (stem cell markers) and CD90 genes (stem cell markers) was significant. The expression of Col-1 and ALP which are primary markers of osteogenic differentiation were significantly reduced to one-fifth and one-tenth compared to the

Table 3
The experimental groups of cells exposed to different concentrations of CGF.

I	H	G	F	E	D	C	B	A	Group
µg/mL	µg/mL	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	Unit
375	750	1.56	3.12	6.25	12.5	25	50	100	Concentration
R	**Q**	**P**	**O**	**N**	**M**	**L**	**K**	**J**	**Group**
ng/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	Unit
750	1.5	3	6	11.75	23.5	47	94	187	Concentration

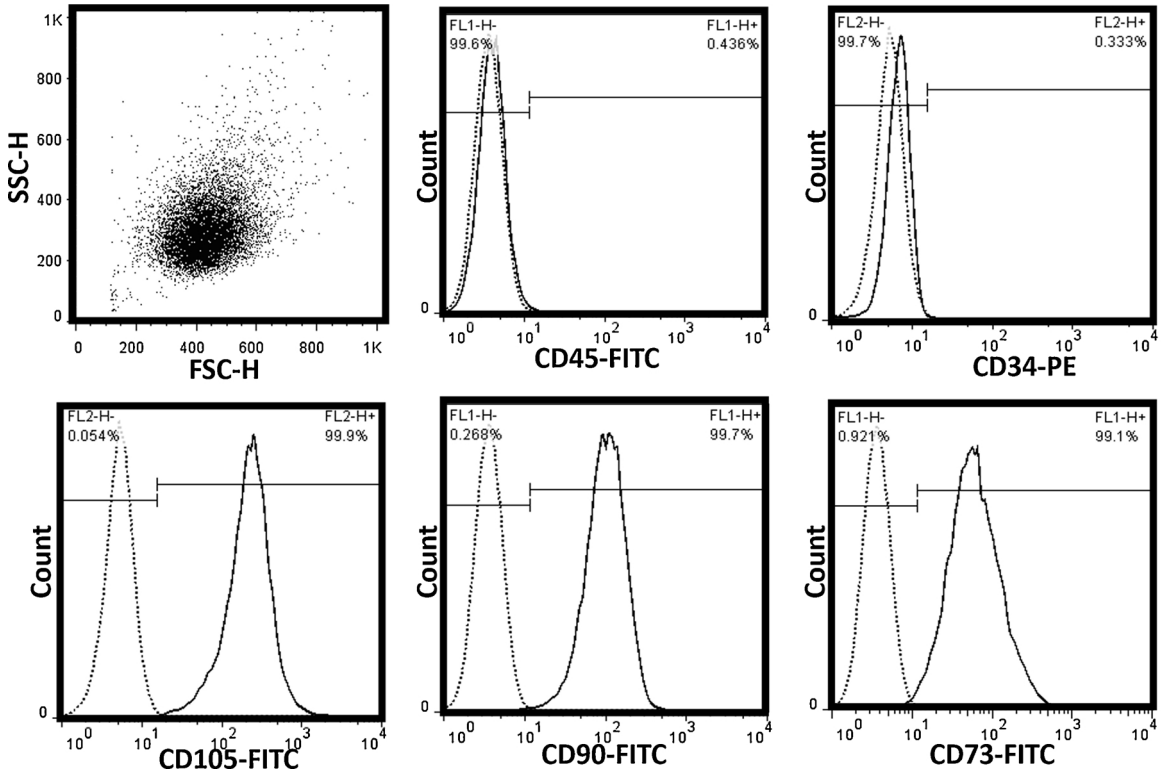

Fig. 1. Flow cytometry analysis showing positive expression of CD90, CD105, CD73, and negative expression of CD45 and CD34.

control. On the other hand, the expression of OSX (Sterix: median osteogenic differentiation marker), OP and OC (late osteogenic differentiation Markers) genes were significantly increased (p-value < 0.05) in cells induced by osteogenic medium compared to control group (1.5, 3.5, and 3.8 times, respectively).

3.2. The optimal concentration of CM

Different concentrations of CM from group A (0.8 g/mL) to T (1.5 μg/mL) were studied in 20 experimental groups. After 48 h exposure, PDLSCs demonstrated a significantly higher proliferation in 6.25 mg/mL concentration of CM (H) than the negative control ($p < 0.05$) and exceeded the positive control ($p < 0.05$). However, high concentrations of CM markedly inhibited the proliferation of PDLCs ($p < 0.05$). The

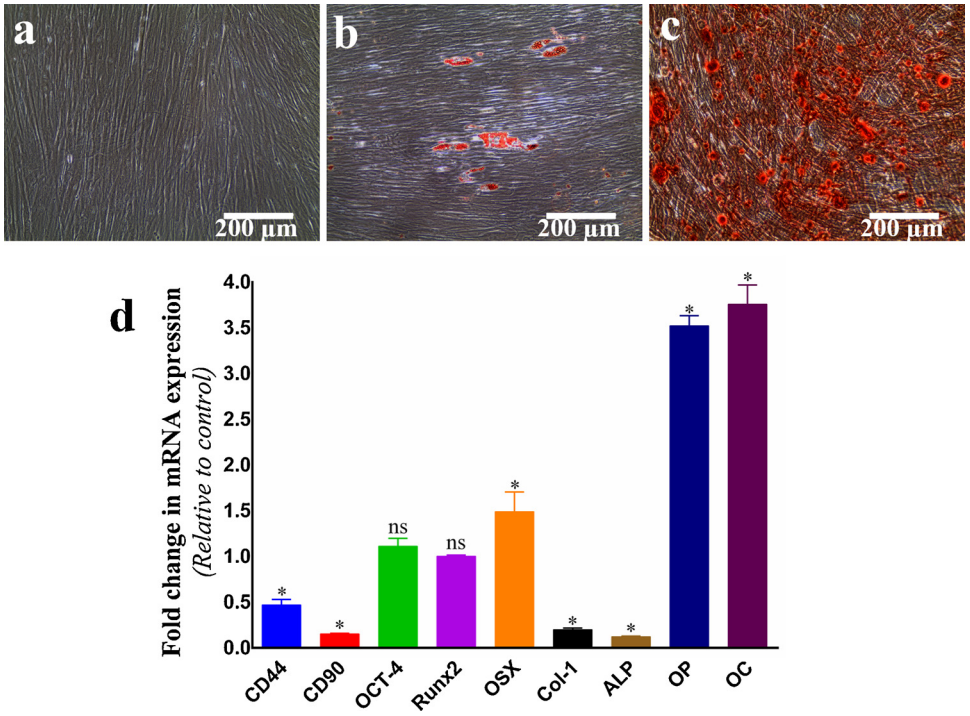

Fig. 2. a) Morphology of isolated PDLSCs; b) Oil Red O staining; c) Alizarin Red S staining ; d) Changes in gene expressions of CD44, CD90, OCT4, RUNX2, OSX, Collagen type 1 (Col-1), alkaline phosphatase (ALP), osteopontin (OP), and osteocalcin (OC) after 2 weeks of culture in the osteogenic medium relative to the control medium (normalized to one). * $p < 0.05$, ns: non-significant. PDLSCs: periodontal ligament stem cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

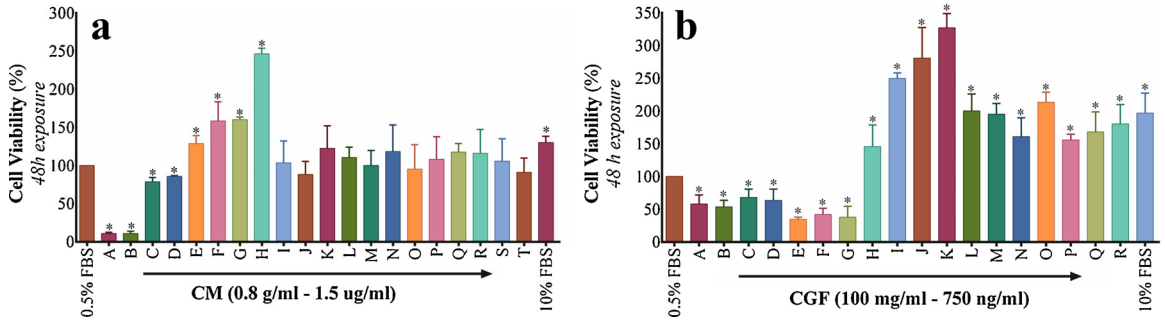

Fig. 3. Cell viability of PDLSCs analyzed by MTT assay after 48 h treatment with CM (conditioned medium) (a) or CGF (concentrated growth factor) (b). Results are presented as mean ± SEM (n = 5 in each group). Control cells were cultured in medium containing 10% or 0.5% FBS. Cell viability was expressed as the percentage of the negative control group (0.5% FBS). * $p < 0.05$.

proliferation rate of the cells treated with a low concentration of CM (I: 3 mg/mL - T: 1.5 µg/mL) was not significantly different from control groups ($p > 0.05$; Fig. 3a).

3.3. The optimal concentration of CGF

The proliferation rate of the PDLSCs treated with some concentration of CGF (I–K) was significantly higher than the positive and negative control groups throughout the 48 h incubation period ($p < 0.05$). However, no dose-dependent effect was found among the experimental groups. Significantly lower cell proliferation than negative control was detected at high doses (A–G) of CGF ($p < 0.05$), while there was no significant difference between the low concentrations (L–R) and positive control ($p > 0.05$). The highest cell proliferation was detected at concentration of 94 µg/mL CGF (K) ($p < 0.05$; Fig. 3b).

3.4. Effect of CGF-CM on PDLSCs proliferation

The influence of the mixture of CGF (94 µg/mL) + CM (6.25 mg/mL) at different percentages was evaluated with a 48-h proliferation assay. The PDLSCs proliferation increased significantly with the increase of the CGF and decrease of CM percentage ($p < 0.05$), exhibiting a dose-response effect. The compound containing 90% CGF-10% CM showed the best results and were significantly different from positive control ($p < 0.05$; Fig. 4a).

Cell proliferation rates at this concentration (90% CGF-10% CM) was also tested at two other time points (24 and 72 h). On the first day, there was no significant difference between group treated with CGF-CM and the negative control ($p > 0.05$). Nevertheless, on the third day, this experimental group had a significant increase in cell number and exceeded positive control ($p < 0.05$; Fig. 4B).

4. Discussion

Regeneration of damaged periodontal tissue requires PDLSCs proliferation and differentiation. In this study, we evaluated the synergistic effect of CGF-CM on PDLSCs proliferation.

4.1. Isolation and characterization of PDLSCs

The periodontal ligament (PDL) is a soft connective tissue located between the cementum and the inner wall of the alveolar bone which contains a cell population that can differentiate into cementoblasts or osteoblasts (Bartold and Gronthos, 2017). PDLSCs isolated from human tissues were first identified in 2004 by Seo et al. (2004). Premolars extracted for orthodontic reasons or impacted third molars can be considered as an appropriate source of PDLSCs. In the present study, we used extracted teeth for orthodontic purposes. Although several studies used impacted third molars for isolation of PDLSCs (Lei et al., 2014; Moshaverinia et al., 2014), Kim and Song isolated these cells from the premolars extracted for orthodontic purposes (Kim et al., 2012; Song et al., 2012).

Isolation of PDLSCs can be successfully performed either by the outgrowth method or by enzymatic digestion (Prateeptongkum et al., 2016). However, different characteristics of cells isolated by enzymatic dissociation in comparison to those isolated by direct outgrowth from tissue explants have been confirmed (Bakopoulou et al., 2011; Hilkens et al., 2013). Because of the extracellular matrix degradation, the enzymatic approach can lead to the isolation of a heterogeneous cell population with lower growth factors (Yoon et al., 2013). In the present

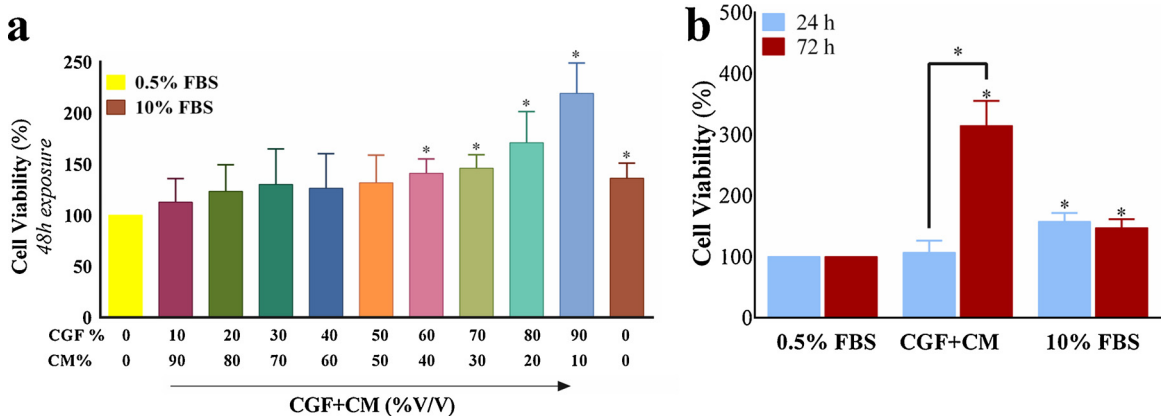

Fig. 4. Cell viability of PDLSCs analyzed by MTT assay after 48 h treatment with different concentration of CGF + CM (A) and after 24 and 72 h exposure to the optimal concentration (90% CGF-10% CM) (B). Results are presented as mean ± SEM (n = 5 in each group). Control cells were cultured in medium containing 10% or 0.5% FBS. Cell viability was expressed as the percentage of the negative control group (0.5% FBS). * $p < 0.05$.

study, the explant method was used for PDLSCs isolation due to the importance of growth factors in the secretome.

We evaluated the MSCs characteristics of isolated cells by assessment of the specific antigens' expression on the cell surface, and their multipotential differentiation capacity. Immunophenotype analysis of specific markers expression in our study demonstrated a high rate of positivity (99%) for the typical mesenchymal stem cell marker proteins, and the absence of hematopoietic markers on tested cells. The positive expression of MSC surface markers in PDLSCs was also reported in previous studies (Bartold and Gronthos, 2017; Lei et al., 2014; Moshaverinia et al., 2014; Seo et al., 2004). We confirmed the multilineage differentiation potential of isolated PDLSCs by Oil Red O and Alizarin Red staining. In RT-PCR analysis, expression of specific stem cell markers (CD44 and CD90) and primary osteoblast differentiation markers (Runx2 and ALP) were significantly reduced after osteogenic induction. In contrast, expression of the final osteoblast differentiation markers (OSX, OP and OC) seen in mature osteoblasts was significantly up-regulated. Differentiation of PDLSCs into osteoblasts, chondrocytes, and adipocytes cells has been also reported in other studies (Kim et al., 2012; Prateeptongkum et al., 2016; Song et al., 2012).

4.2. CM preparation and determination of the optimal concentration

Stem cell secretome is frequently used in the form of conditioned medium (Cianci et al., 2016; Xia et al., 2016). However, it is important to note that secretome derived from various kinds of stem cells may vary in properties, and one secretome might not be effective for the treatment of all conditions (Flower et al., 2015). The quality of CM is also dependent on the method used for its collection. One of the most common methods of stem cells' preconditioning to stimulate growth factor secretion is serum deprivation which can simulate stress conditions during tissue damage (Tran and Damaser, 2015). In order to prepare CM, different studies have used xeno-free chemically defined medium (Rajan et al., 2016), complete media (containing 10% FBS) (Cianci et al., 2016), serum-free media (Ahmed et al., 2016), or DMEM supplemented with 0.05% bovine serum albumin (Du et al., 2013), 1% FBS (Al-Sharabi et al., 2017), or 2% FBS (Jin et al., 2016). In this study, we used 0.5% FBS preconditioning based on the study of Paschalidis et al. who compared CMs collected from DPSC under normal serum (15% FBS) and serum deprivation conditions (0.5% FBS). The results of their study showed that CM collected under 0.5% FBS had more positive effects than those collected under normal serum (Paschalidis et al., 2014).

The optimal time-points of CM collection is also the subject of many contradictions in the literature. Condition media is often collected within the first hours or days after stress induction (Chen et al., 2008; Lee et al., 2012; Oskowitz et al., 2011; Osugi et al., 2012). Yamaguchi et al., and Shimojima et al., collected the condition media of stem cells after 48 h by centrifugation of the media for 3 min at 440× *g* and 3 min at 1740× *g* (Yamaguchi et al., 2015); Whereas Cianci et al. collected the CM of periodontal ligament stem cells at 24, 48 and 72 h (Cianci et al., 2016). In the present study, CM was collected 48 h after stress induction and freeze-dried. Lyophilization of the CM could help in providing more accurate concentrations and preserving the secreted factors' activity (Yao et al., 2016).

Since CM include a broad range of cytokines or other materials, predicting the optimal concentration of CM in term of cell proliferation is very important. We investigated the effect of different concentration of CM on the proliferation of PDLSCs. Xia et al. also assessed the effects of PDLSCs-CM on PDLSCs (Xia et al., 2016). Our study showed that high and low concentrations of lyophilized CM may cause a significant decrease in cell proliferation. Our result agrees with the result of Yao et al. who prepared freeze-dried CM from MSCs and investigated its effect on neural stem cells. The concentration gradient assay performed with the MSC-Derived CM from 50 ng/mL to 500 ng/mL, revealed that the optimal concentration should be 250 ng/mL (Yao et al., 2016). Although, Park et al. who examined different concentrations of lyophilized-CM (1, 5, and 10 μg/mL) showed a dose-dependent manner in its effect (Park et al., 2016), Paschalidis et al., evaluated two different concentrations (50% and 100%) of fresh CM on DPSCs proliferation and documented a stronger result for the 50% concentration (Paschalidis et al., 2014). Achieving an optimum balance between metabolic inhibitory effect of by-products and the positive effect of paracrine factors is the probable explanation for the effect of a certain concentration of CM (Hu et al., 2013).

4.3. CGF preparation and determination of the optimal concentration

CGF is a platelet-concentrate and a rich source of growth factors, which has been introduced by Sacco with an easy and rapid one-step preparation (Rodella et al., 2011). Since long-term growth factors stability is better maintained by freeze-drying, we preferred using a freeze-dried CGF. Li et al. by comparing lyophilized Platelet-rich fibrin and commonly used fresh Platelet-rich fibrin approved the high performance of the lyophilized form (Li et al., 2014).

The effect of CGF on cell proliferation has already been fully explored in a review article (Tabatabaei et al., 2020). Rich storage of growth factors responsible for cell proliferation in CGF and protection of these factors by CGF dense network can explain the behavior of cells treated with this product. In the present study, high concentrations of CGF had an inhibitory effect on cell proliferation. This result is in agreement with studies that showed the negative effect of a high dose of CGF on cell proliferation (Chen et al., 2018; Honda et al., 2013; Hong et al., 2019; Qiao and An, 2017). The negative role of the high dose of CGF may be due to the presence of a high concentration of TGF-β. Unfavorable effects of high concentration of this growth factor has been previously demonstrated (Tabatabaei and Torshabi, 2016). The high amount of proteolytic enzymes found in high concentrations of platelet derivatives could also explain their inhibitory effect on cell proliferation (Stessuk et al., 2016).

Our result is in disagreement with studies that confirmed proliferation of cells treated with CGF in a dose-dependent manner (Jin et al., 2018; Yu and Wang, 2014). The difference in platelet count of CGF, blood volume used for CGF preparation, and the type of CGF could explain these controversies (Tabatabaei et al., 2020).

4.4. Effect of CGF-CM compound on PDLSCs

The mixture of stem cells and platelet derivatives has so far been investigated in some studies. Yamada et al. used a combination of PRP and MSCs and confirmed the usefulness of the MSCs/PRP mixture in osteogenesis (Yamada et al., 2004). In the study of Zhao et al., the PDLSCs/PRF construct showed a more effective outcome than the control group (Zhao et al., 2013). Also, Chen et al. who combined the BMSCs with CGF, reported superior osteoinductive activity of the CGF + BMSC combination (Chen et al., 2018).

Despite extensive research on either CGF or CM, no study has yet examined the effect of their combination. Stessuk et al. investigated the effect of PRP and adipose-derived stem cells-CM on fibroblast and keratinocyte proliferation and showed the significant proliferation of fibroblast cells in the presence of 25% PRP and 25% CM, while keratinocyte proliferation after exposure to different concentration of PRP-CM was not significantly different from negative control (serum-free media) (Stessuk et al., 2016). We examined the effect of different mixture of CGF-CM on PDLSCs proliferation. In our study, the compound of 90% CGF and 10% CM demonstrated greater efficacy compared with other combinations. The differences between the CGF and PRP, the source of stem cells, and the method of CM preparation could be a possible explanation for the difference in the percentages mentioned in the above study compared to our study. Further studies on other cells such as immune cells, osteoblasts, and fibroblasts, which are present in the damaged tissue are warranted to clarify the benefits of

CGF-CM compound.

5. Conclusion

In summary, the optimal concentrations of lyophilized CM and CGF was investigated by examining a wide range of different concentrations of these two substances alone and in combination. The results of this study show that CGF and CM at a certain concentration exert a proliferative effect on cells. Their combination will exhibit a promotive effect with the increase of the CGF and decrease of CM percentage. The synergistic effect of this compound can further be investigated on other cells presented in the periodontal ligament for using it as a product for periodontal regeneration.

Authors contributions

F. Tabatabaei conceived the ideas and designed the experiments. Z. Aghamohamadi, M. Kadkhodazadeh, M. Torshabi, F. Tabatabaei performed the experiments, analyzed, and interpreted the data. Z. Aghamohamadi, F. Tabatabaei wrote the paper and all authors have contribution in revising it and approving the final version.

Funding

This study was supported by Research Institute of Dental Sciences, Shahid Beheshti University of Medical Sciences (#11583).

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

References

Acebes-Huerta, A., Arias-Fernandez, T., Bernardo, A., Munoz-Turrillas, M.C., Fernandez-Fuertes, J., Seghatchian, J., Gutierrez, L., 2019. Platelet-derived bio-products: classification update, applications, concerns and new perspectives. Transfus. Apher. Sci., 102716.

Ahmed, N.E., Murakami, M., Kaneko, S., Nakashima, M., 2016. The effects of hypoxia on the stemness properties of human dental pulp stem cells (DPSCs). Sci. Rep. 6, 35476.

Al-Sharabi, N., Mustafa, M., Ueda, M., Xue, Y., Mustafa, K., Fristad, I., 2017. Conditioned medium from human bone marrow stromal cells attenuates initial inflammatory reactions in dental pulp tissue. Dent. Traumatol. 33, 19–26.

Bakopoulou, A., Leyhausen, G., Volk, J., Tsiftsoglou, A., Garefis, P., Koidis, P., Geurtsen, W., 2011. Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth. Calcif. Tissue Int. 88, 130–141.

Bartold, P.M., Gronthos, S., 2017. Standardization of criteria defining periodontal ligament stem cells. J. Dent. Res. 96, 487–490.

Carmagnola, D., Pellegrini, G., Dellavia, C., Rimondini, L., Varoni, E., 2019. Tissue engineering in periodontology: biological mediators for periodontal regeneration. Int. J. Artif. Organs 42, 241–257.

Chen, L., Tredget, E.E., Wu, P.Y., Wu, Y., 2008. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE 3, e1886.

Chen, X., Wang, J., Yu, L., Zhou, J., Zheng, D., Zhang, B., 2018. Effect of concentrated growth factor (CGF) on the promotion of osteogenesis in bone marrow stromal cells (BMSC) in vivo. Sci. Rep. 8, 5876.

Cianci, E., Recchiuti, A., Trubiani, O., Diomede, F., Marchisio, M., Miscia, S., Colas, R.A., Dalli, J., Serhan, C.N., Romano, M., 2016. Human periodontal stem cells release specialized proresolving mediators and carry immunomodulatory and prohealing properties regulated by lipoxins. Stem Cells Transl. Med. 5, 20–32.

Du, Z., Wei, C., Cheng, K., Han, B., Yan, J., Zhang, M., Peng, C., Liu, Y., 2013. Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. J. Surg. Res. 183, 907–915.

Flower, T., Pulsipher, V., Moreno, A., 2015. A new tool in regenerative medicine: mesenchymal stem cell secretome. J. Stem Cell Res. Ther. 1, 1–3.

Fretwurst, T., Larsson, L., Yu, S.H., Pilipchuk, S.P., Kaigler, D., Giannobile, W.V., 2018. Periodontal tissue bioengineering: is the future now? Compend. Cont. Educ. Dent. 39, 218–223 quiz 224.

Hilkens, P., Gervois, P., Fanton, Y., Vanormelingen, J., Martens, W., Struys, T., Politis, C., Lambrichts, I., Bronckaers, A., 2013. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell Tissue Res. 353, 65–78.

Honda, H., Tamai, N., Naka, N., Yoshikawa, H., Myoui, A., 2013. Bone tissue engineering with bone marrow-derived stromal cells integrated with concentrated growth factor in Rattus norvegicus calvaria defect model. J. Artif. Organs 16, 305–315.

Hong, S., Li, L., Cai, W., Jiang, B., 2019. The potential application of concentrated growth factor in regenerative endodontics. Int. Endod. J. 52, 646–655.

Hu, L., Zhao, J., Liu, J., Gong, N., Chen, L., 2013. Effects of adipose stem cell-conditioned medium on the migration of vascular endothelial cells, fibroblasts and keratinocytes. Exp. Ther. Med. 5, 701–706.

Jin, Z., Feng, Y., Liu, H., 2016. Conditioned media from differentiating craniofacial bone marrow stromal cells influence mineralization and proliferation in periodontal ligament stem cells. Hum. Cell 29, 162–175.

Jin, R., Song, G., Chai, J., Gou, X., Yuan, G., Chen, Z., 2018. Effects of concentrated growth factor on proliferation, migration, and differentiation of human dental pulp stem cells in vitro. J. Tissue Eng. 9. https://doi.org/10.1177/2041731418817505.

Kawaguchi, H., Hirachi, A., Hasegawa, N., Iwata, T., Hamaguchi, H., Shiba, H., Takata, T., Kato, Y., Kurihara, H., 2004. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. J. Periodontol. 75, 1281–1287.

Kim, S.S., Kwon, D.-W., Im, I., Kim, Y.-D., Hwang, D.-S., Holliday, L.S., Donatelli, R.E., Son, W.-S., Jun, E.-S., 2012. Differentiation and characteristics of undifferentiated mesenchymal stem cells originating from adult premolar periodontal ligaments. Korean J. Orthod. 42, 307–317.

Lee, S.H., Jin, S.Y., Song, J.S., Seo, K.K., Cho, K.H., 2012. Paracrine effects of adipose-derived stem cells on keratinocytes and dermal fibroblasts. Ann. Dermatol. 24, 136–143.

Lei, M., Li, K., Li, B., Gao, L.-N., Chen, F.-M., Jin, Y., 2014. Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation. Biomaterials 35, 6332–6343.

Li, Q., Reed, D.A., Min, L., Gopinathan, G., Li, S., Dangaria, S.J., Li, L., Geng, Y., Galang, M.-T., Gajendrareddy, P., Zhou, Y., Luan, X., Diekwisch, T.G.H., 2014. Lyophilized platelet-rich fibrin (PRF) promotes craniofacial bone regeneration through Runx2. Int. J. Mol. Sci. 15, 8509–8525.

Moshaverinia, A., Xu, X., Chen, C., Ansari, S., Zadeh, H.H., Snead, M.L., Shi, S., 2014. Application of stem cells derived from the periodontal ligament or gingival tissue sources for tendon tissue regeneration. Biomaterials 35, 2642–2650.

Muhammad, S.A., Nordin, N., Fakurazi, S., 2018. Regenerative potential of secretome from dental stem cells: a systematic review of preclinical studies. Rev. Neurosci. 29, 321–332.

Oskowitz, A., McFerrin, H., Gutschow, M., Carter, M.L., Pochampally, R., 2011. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. Stem Cell Res. 6, 215–225.

Osugi, M., Katagiri, W., Yoshimi, R., Inukai, T., Hibi, H., Ueda, M., 2012. Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng. Part A 18, 1479–1489.

Park, C.M., Kim, M.J., Kim, S.M., Park, J.H., Kim, Z.H., Choi, Y.S., 2016. Umbilical cord mesenchymal stem cell-conditioned media prevent muscle atrophy by suppressing muscle atrophy-related proteins and ROS generation. In Vitro Cell. Dev. Biol. Anim. 52, 68–76.

Paschalidis, T., Bakopoulou, A., Papa, P., Leyhausen, G., Geurtsen, W., Koidis, P., 2014. Dental pulp stem cells' secretome enhances pulp repair processes and compensates TEGDMA-induced cytotoxicity. Dent. Mater. 30, e405–e418.

Pawitan, J.A., 2014. Prospect of stem cell conditioned medium in regenerative medicine. Biomed Res. Int. 2014. https://doi.org/10.1155/2014/965849.

Prateeptongkum, E., Klingelhöffer, C., Müller, S., Ettl, T., Morsczeck, C., 2016. Characterization of progenitor cells and stem cells from the periodontal ligament tissue derived from a single person. J. Periodont. Res. 51, 265–272.

Qiao, J., An, N., 2017. Effect of concentrated growth factors on function and Wnt3a expression of human periodontal ligament cells in vitro. Platelets 28, 281–286.

Rajan, T.S., Giacoppo, S., Trubiani, O., Diomede, F., Piattelli, A., Bramanti, P., Mazzon, E., 2016. Conditioned medium of periodontal ligament mesenchymal stem cells exert anti-inflammatory effects in lipopolysaccharide-activated mouse motoneurons. Exp. Cell Res. 349, 152–161.

Ren, X., Zhao, M., Lash, B., Martino, M.M., Julier, Z., 2019. Growth factor engineering strategies for regenerative medicine applications. Front. Bioeng. Biotechnol. 7, 469.

Rodella, L.F., Favero, G., Boninsegna, R., Buffoli, B., Labanca, M., Scari, G., Sacco, L., Batani, T., Rezzani, R., 2011. Growth factors, CD34 positive cells, and fibrin network analysis in concentrated growth factors fraction. Microsc. Res. Tech. 74, 772–777.

Seo, B.-M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahim, J., Young, M., Robey, P.G., Wang, C.Y., Shi, S., 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364, 149–155.

Song, M., Kim, H., Choi, Y., Kim, K., Chung, C., 2012. Skeletal myogenic differentiation of human periodontal ligament stromal cells isolated from orthodontically extracted premolars. Korean J. Orthod. 42, 249–254.

Stessuk, T., Puzzi, M.B., Chaim, E.A., Alves, P.C., de Paula, E.V., Forte, A., Izumizawa, J.M., Oliveira, C.C., Frei, F., Ribeiro-Paes, J.T., 2016. Platelet-rich plasma (PRP) and adipose-derived mesenchymal stem cells: stimulatory effects on proliferation and migration of fibroblasts and keratinocytes in vitro. Arch. Dermatol. Res. 308, 511–520.

Tabatabaei, F.S., Torshabi, M., 2016. Effects of non-collagenous proteins, TGF-beta1, and PDGF-BB on viability and proliferation of dental pulp stem cells. J. Oral Maxillofac. Res. 7, e4.

Tabatabaei, F., Aghamohammadi, Z., Tayebi, L., 2020. In vitro and in vivo effects of concentrated growth factor on cells and tissues. J. Biomed. Mater. Res. A 108, 1338–1350. https://doi.org/10.1002/jbm.a.36906.

Tran, C., Damaser, M.S., 2015. Stem cells as drug delivery methods: application of stem cell secretome for regeneration. Adv. Drug Deliv. Rev. 82–83, 1–11.

Xia, Y., Tang, H.N., Wu, R.X., Yu, Y., Gao, L.N., Chen, F.M., 2016. Cell responses to conditioned media produced by patient-matched stem cells derived from healthy and

inflamed periodontal ligament tissues. J. Periodontol. 87, e53–63.
Xu, X.Y., Li, X., Wang, J., He, X.T., Sun, H.H., Chen, F.M., 2019. Concise review: periodontal tissue regeneration using stem cells: strategies and translational considerations. Stem Cells Transl. Med. 8, 392–403.
Yamada, Y., Ueda, M., Naiki, T., Takahashi, M., Hata, K., Nagasaka, T., 2004. Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: tissue-engineered bone regeneration. Tissue Eng. 10, 955–964.
Yamaguchi, S., Shibata, R., Yamamoto, N., Nishikawa, M., Hibi, H., Tanigawa, T., Ueda, M., Murohara, T., Yamamoto, A., 2015. Dental pulp-derived stem cell conditioned medium reduces cardiac injury following ischemia-reperfusion. Sci. Rep. 5, 16295.
Yao, Y., Huang, C., Gu, P., Wen, T., 2016. Combined MSC-Secreted factors and neural stem cell transplantation promote functional recovery of PD rats. Cell Transplant 25, 1101–1113.
Yoon, J.H., Roh, E.Y., Shin, S., Jung, N.H., Song, E.Y., Chang, J.Y., Kim, B.J., Jeon, H.W., 2013. Comparison of explant-derived and enzymatic digestion-derived MSCs and the growth factors from wharton jelly. Biomed. Res. Int. 2013, 8.
Yu, B., Wang, Z., 2014. Effect of concentrated growth factors on beagle periodontal ligament stem cells in vitro. Mol. Med. Rep. 9, 235–242.
Zhao, Y.-H., Zhang, M., Liu, N.-X., Lv, X., Zhang, J., Chen, F.-M., Chen, Y.-J., 2013. The combined use of cell sheet fragments of periodontal ligament stem cells and platelet-rich fibrin granules for avulsed tooth reimplantation. Biomaterials 34, 5506–5520.
Zhu, W., Liang, M., 2015. Periodontal ligament stem cells: current status, concerns, and future prospects. Stem Cells Int. 2015, 972313.