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ORIGINAL ARTICLE

Effect of lipopolysaccharide on cell proliferation and vascular endothelial growth factor secretion of periodontal ligament stem cells

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KEYWORDS

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Abstract *Purpose:* Periodontal ligament stem cells (PDLSCs) have considerable potential for use as a means of achieving periodontal regeneration due to their noteworthy proliferative properties and secretory functions. In particular, PDLSCs secrete vascular endothelial growth factor (VEGF) which enhances angiogenesis and osteogenesis. The resulting repair and development of blood vessels and hard tissues which would occur in the presence of these cells could be central to an effective periodontal regeneration procedure.

The bacterial biofilm of tooth surface related to the periodontium might provide either an inhibition or a stimulus to different factors involved in a regenerative process. Cell culture experiments have been investigated *in vitro* by adding lipopolysaccharide (LPS) to the culture medium but the effect of various concentration of LPS in these circumstances has not been investigated. Therefore, this study aimed to investigate the effect of LPS concentrations on proliferation of PDLSCs *in vitro* and on their secretion of VEGF.

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Materials and methods: PDLSCs were treated with 0, 5, 10 and 20 μg/mL of *Escherichia coli* LPS. At 48 and 96 h, total cell numbers of control and LPS treated PDLSCs were counted by haemocytometer under a microscope. The VEGF concentration in the conditioned media of the PDLSCs was measured by ELISA.

Results: Rate of cell proliferation of PDLSCs decreased significantly in all LPS treated groups at both 48 h and 96 h except for the group treated with 5 μg/mL of LPS at 48 h. At both 48 and 96 h, VEGF secretion from PDLSCs was reduced significantly at all three LPS concentrations. There was no statistically significant difference in cell proliferation and the amount of VEGF secretion of PDLSCs among the groups treated with different LPS concentrations. No statistically significant change was found in cell proliferation of LPS treated PDLSCs over time, whereas VEGF secretion of PDLSCs was found to have increased significantly with time despite the LPS treatment.

Conclusions: LPS reduced cell proliferation and VEGF secretion of PDLSCs, suggesting that periodontal pathogens might reduce the capability of PDLSCs in periodontal regeneration. Yet, LPS treated PDLSCs remained viable and VEGF secretion increased significantly over time. Further research is needed to study the potential use of PDLSCs in periodontal regeneration and the relationship of biofilm LPS accumulations.

1. Introduction

Chronic Periodontal Disease is the chronic inflammation of periodontium resulting in tissue destruction that can eventually lead to tooth loss (Hosokawa et al., 2005). According to the National Oral Health Survey of Adults, 94% of dentate adults in Malaysia have periodontal disease (Oral Health Division, 2010). Dentists have succeeded in controlling periodontitis through conventional therapies but have failed thus far to find satisfactory methods to restore the lost periodontal tissues (Zhu and Liang, 2015). Complete regeneration of periodontal tissues following the periodontal disease at present seems a distant goal despite the extensive research (Siaili et al., 2018). However, novel stem cell-based therapeutics may provide a method of completely and reliably reconstituting tissues damaged by periodontal disease (Chen et al., 2012). Intensive research in the last decade involving other tissues and organs in the body indicate that tissue engineering is able to regenerate tissues in a beneficial manner (Jansen, 2012).

Periodontal ligament stem cells (PDLSCs) are an appropriate cell source for functional periodontal tissue regeneration as they possess unique periodontal regenerative capacities (Li et al., 2014). They also have low immunogenicity and additionally an immunosuppressive property which acts on T and B cells which should facilitate the use of allogeneic PDLSCs for periodontal regeneration purposes (Zhu and Liang, 2015). Additionally, PDLSCs are highly proliferative and have a therapeutic potential for reconstruction of tissues destroyed by periodontal disease (Zheng et al., 2015). The feasibility of using PDLSCs in regenerative dentistry depends on whether growth is affected by bacterial virulence factors (Albiero et al., 2015). It has been suggested expansion of PDLSCs within a diseased periodontium may accelerate periodontal regeneration (Cianci et al., 2016).

The periodontal regeneration process may be enhanced by incorporating signaling molecules that modulate cell functions (Ammar et al., 2018). PDLSCs produce a wide variety of cytokines to maintain periodontal tissue homeostasis including vascular endothelial growth factor (VEGF) (Kittaka et al., 2013). VEGF is a potent mitogenic growth factor that initiates endothelial cell proliferation and blood vessel formation (Kittaka et al., 2013). Promotion of neovascularization during periodontal regeneration is necessary because the newly formed blood vessels supply oxygen, nutrition and progenitor cells to the damaged region, thereby facilitating the regenerative process (Kittaka et al., 2013). VEGF may play a key role by enhancing angiogenesis and osteogenesis for regeneration (Lee et al., 2012).

The periodontal microenvironment is constantly interacting with the bacterial biofilm present on the adjacent tooth surfaces that can affect PDLSCs properties and functional abilities (Chatzivasileiou et al., 2015). The biofilm of the diseased periodontium is composed of a complex microbiota, predominantly of anaerobic Gram-negative bacteria (Souto et al., 2006). Lipopolysaccharide (LPS), an endotoxin in Gram-negative bacteria such as *Escherichia coli (E. coli)* is an important triggering factor of periodontitis (Ambili et al., 2017). When in contact with host immune cells as well as gingival fibroblasts, LPS induces an immune-inflammatory response characterized by the release of pro-inflammatory cytokines (Albiero et al., 2015).

According to Maidwell-Smith et al. (1987), the amount of LPS on the root surface of periodontally-involved teeth ranges from 19 to 394 ng per tooth. The mere presence of periodontal pathogens or their endotoxins is not sufficient to cause periodontitis, but when concentrations pass a certain threshold level, they become tissue-damaging (Newman and Takei, 2012). *Porphyromonas gingivalis (P. gingivalis)* is recognized as a major pathogen in the development and progression of periodontitis (Palaska et al., 2016). *E. coli* LPS was investigated in this study because it has higher endotoxic potency and induces higher inflammatory signalling than *P. gingivalis* (Chang et al., 2013; Chatzivasileiou et al., 2013). The lipid A molecule of *E. coli* LPS is similar in structure to that of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum,* both of which are associated with periodontitis (Jain and Darveau, 2010).

Studies on the effects of various LPS concentrations that could mimic the biofilm action on the biological properties of PDLSCs are inconclusive and interpretation of the results

is controversial. Chen et al. (2012) demonstrated that in the diseased periodontium, tissue repair does not occur naturally due to a lack of robust stem cells. *E. coli* LPS has been shown to inhibit the proliferation of dental follicle cells (DFCs), whereas *P. gingivalis* LPS inhibits the proliferation of both PDLSCs and DFCs (Lan et al., 2013; Morsczeck et al., 2012). In contrast, in a study done by Albiero et al. (2015), PDLSCs treated with *E. coli* LPS remained proliferative regardless of LPS concentration. Other studies evaluating the effect of *E. Coli* LPS on DFCs and adipose-derived stem cells have also documented increased proliferation (Hwa Cho et al., 2006; Martinez et al., 2012; Morsczeck et al., 2012). Inflamed and healthy PDLSCs cultured together have shown enhanced cell proliferation compared to purely healthy PDLSCs (Tang et al., 2016).

VEGF secretion from PDLSCs is also worthy of study because of its potential for enhancing periodontal regeneration. Palaska et al. (2016) reported that *E. coli* LPS induced a significant release of VEGF from mast cells and the effect was not concentration-dependent. Based on the findings of previous research, the current study hypothesized that LPS would inhibit both cell proliferation and VEGF secretion of PDLSCs. To test this hypothesis, cell proliferation and VEGF secretion of PDLSCs were investigated in the presence of 0, 5, 10 and 20 μg/mL of *E. coli* LPS.

2. Material and methods

2.1. Materials

KnockOut™ Dulbecco's Modified Eagle's medium (Gibco, USA), foetal bovine serum (Gibco, USA), GlutaMAX™ L-glutamine (Gibco, USA), penicillin-streptomycin (Gibco, USA), basic fibroblast growth factor (Gibco, USA), Dulbecco's Phosphate-Buffered Saline (Gibco, USA), TrypLE™ Express trypsin (Gibco, USA), LPS from *E. Coli* O111:B4 (Sigma–Aldrich, USA), Trypan blue (Gibco, USA) and Human VEGF Quantikine ELISA plate (R&D Systems, USA) were used.

2.2. Culture and maintenance of PDLSCs

PDLSCs isolated from healthy periodontal tissue were used in this study. Briefly, the cells were isolated using the standard method (Zhu and Liang, 2015) by scraping the periodontal ligament tissues on the roots of the freshly extracted teeth for orthodontic purposes. The tissue obtained was digested with freshly prepared collagenase type IV (Invitrogen, USA) solution at 1 mg/ml concentration and incubated for 30 min. The cells were then centrifuged and suspended in Dulbecco's Modified Eagle's medium (Gibco, USA) supplemented with foetal bovine serum (Gibco, USA), GlutaMAX™ L-glutamine (Gibco, USA), penicillin-streptomycin (Gibco, USA) and basic fibroblast growth factor (Gibco, USA). The cells were seeded in plastic plates. Floating cells were removed after 72 h and the adherent cells were expanded and cryopreserved. These cells were also characterized expressing surface markers CD105, CD73, CD166, CD90 and were negative for CD34, CD45 and HLA-DR (Spoorthi Ravi Banavar, unpublished results, personal communication). Cryopreserved PDLSCs at passage 4 were thawed at 37 °C in a water bath. The cell suspension was centrifuged at 1200 rpm for 5 min. The cell pellet of PDLSCs was re-suspended and transferred to T-75 flasks containing KnockOut™ Dulbecco's Modified Eagle's medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA), 1% GlutaMAX™ L-glutamine (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) and basic fibroblast growth factor (Gibco, USA). PDLSCs were cultured at 37 °C in a 5% CO_2 incubator. Culture medium was replaced every 72 h and cellular confluence was monitored microscopically.

2.3. Plating of PDLSCs and treatment with various LPS concentrations

When PDLSCs reached 80% confluence, they were washed with Dulbecco's Phosphate-Buffered Saline (Gibco, USA) and dissociated with TrypLE™ Express trypsin (Gibco, USA). A single-cell suspension of PDLSCs was seeded onto 6-well plates at 96,000 cells per well and was allowed to attach overnight. PDLSCs were then treated with LPS derived from *E. Coli* O111:B4 (Sigma–Aldrich, USA) at a concentration of 5, 10 and 20 μg/mL for 48 and 96 h. A control group of PDLSCs not treated with LPS was maintained.

2.4. Measurement of cell proliferation

At 48 and 96 h, control and LPS treated PDLSCs were washed, trypsinized and counted by using haemocytometer under a microscope. 0.4% Trypan blue (Gibco, USA) was used to stain dead cells for direct cell counting. The total cell number was calculated in each sample from each well and the average was calculated for each group.

2.5. Measurement of VEGF secretion

At 48 and 96 h, conditioned media were collected from control and LPS treated groups, filtered and stored at −80 °C for VEGF ELISA. Briefly, the samples of conditioned media were added to the wells of Human VEGF Quantikine ELISA plate (R&D Systems, USA) and were further processed as per manufacturer instruction. Human VEGF was quantified by using a spectrophotometer and unknown values were determined by using the standard curve method.

2.6. Statistical analysis

All experiments were performed in triplicate. Data of cell proliferation and VEGF concentration was presented as mean and standard deviation (SD). Data were analysed using One-way ANOVA with post-hoc Tukey HSD test and two-sample t-tests assuming unequal variances. $P < 0.05$ represented a statistically significant difference.

3. Results

3.1. Cell proliferation of PDLSCs

In the control group of PDLSCs, a significant increase of cell count ($P < 0.05$) was observed at 48 and 96 h (Table 1, Fig. 1a and e) while all LPS treated groups showed a non-significant

Table 1 Average total number of viable PDLSCs per well for all LPS concentrations at 48 and 96 h.

Duration of culture (hours)	Average total number of PDLSCs ± SD (1 0 ^{4})				
	0 μg/mL	5 μg/mL	10 μg/mL	20 μg/mL	*P* value
0	9.6×10^4				
48	17.5 ± 0.87	12.42 ± 4.11	9.83 ± 1.51	10.08 ± 1.66	0.013*
96	23.92 ± 1.13	15.83 ± 3.79	11.00 ± 3.12	11.08 ± 2.50	0.002*
P value	0.001*	0.175	0.300	0.302	

* Statistically significant difference compared to the baseline.

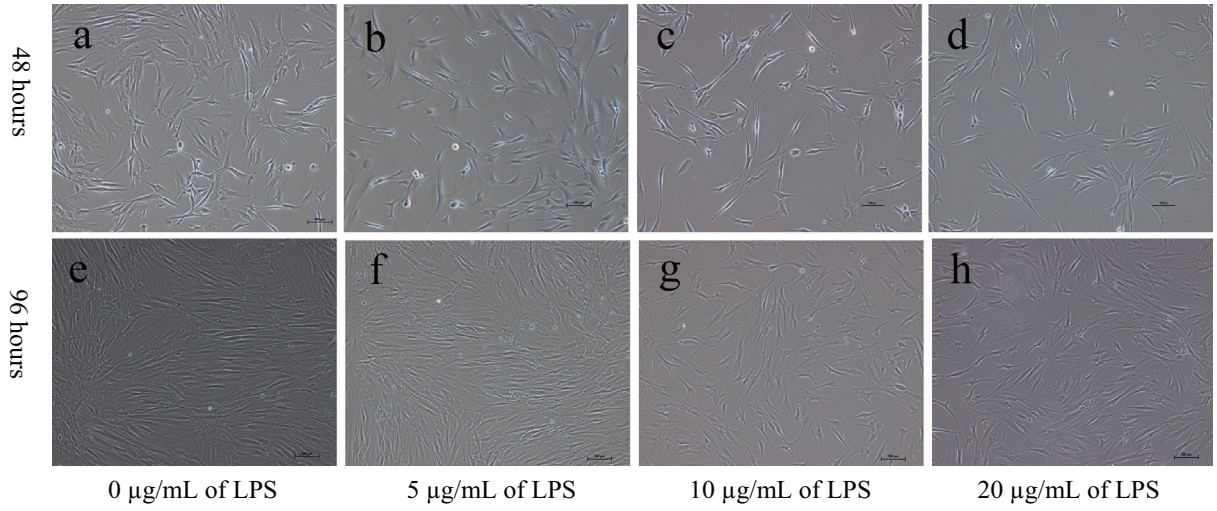

Fig. 1 Microscopic pictures of PDLSCs treated with different LPS concentration at 48 and 96 h. Magnification ×10. All groups of LPS treated PDLSCs retained the capacity to form fibroblast spindle-shape. (a, e) PDLSCs without LPS treatment at 48 and 96 h. (b, f) PDLSCs treated with 5 μg/mL of LPS at 48 and 96 h. (c, g) PDLSCs treated with 10 μg/mL of LPS at 48 and 96 h. (d, h) PDLSCs treated with 20 μg/mL of LPS at 48 and 96 h.

increase in cell proliferation over time (Table 1, Fig. 1b-d and 1f-h). As compared to control, the cell number of PDLSCs was significantly reduced in all LPS treated groups at 48 h and 96 h ($P < 0.05$) except for 5 μg/mL of LPS at 48 h (Table 2). However, there was no time-dependent decrease in the cell number of LPS treated groups (Table 1, Fig. 1b-d and f-h). Besides, there was no significant difference in the cell count among the groups treated with different LPS concentration (Table 2).

Table 2 ANOVA post hoc Tukey test on the average total number of viable PDLSCs per well.

LPS concentration (μg/mL)		*P* value	
		48 h	96 h
0	5	0.108	0.032*
	10	0.017*	0.002*
	20	0.020*	0.002*
5	10	0.560	0.218
	20	0.633	0.229
10	20	0.999	1.000

* Statistically significant difference compared to the baseline.

3.2. VEGF secretion from PDLSCs

Both the control and LPS treated groups showed a significant increase in VEGF concentration at 96 h compared to 48 h ($P < 0.05$), indicating a time-dependent increase in VEGF secretion regardless of the LPS concentration (Table 3). As compared to control, VEGF concentration was significantly lower in LPS treated groups at all LPS concentrations at both time points ($P < 0.05$) (Table 4). There was no significant difference in VEGF concentration among the groups treated with different LPS concentration except at 48 h between 10 and 20 μg/mL of LPS (Table 4).

4. Discussion

The stem cell mediated periodontal regeneration occurring in the periodontal microenvironment is constantly affected by bacterial interaction. During periodontal regeneration, PDLSCs proliferate and actively secrete cytokines and growth factors to promote the differentiation of progenitor cells and to restore PDL, cementum and alveolar bone (Chen et al., 2012; Baraniak and McDevitt, 2010). For instance, VEGF, a

Table 3 VEGF concentration secreted by PDLSCs per well for all LPS concentrations at 48 and 96 h.

Duration of culture (hours)	Average VEGF concentration ± SD (pg/mL)				
	0 μg/mL	5 μg/mL	10 μg/mL	20 μg/mL	*P* value
0	Non-applicable				
48	1340.13 ± 57.79	578.19 ± 20.40	683.66 ± 43.30	611.05 ± 32.87	0.000*
96	1751.34 ± 110.07	1211.12 ± 80.93	1166.71 ± 81.32	1351.48 ± 134.18	0.000*
P value	0.010*	0.000*	0.010*	0.000*	

* Statistically significant difference compared to the baseline.

Table 4 ANOVA post hoc Tukey test on VEGF concentration of PDLSCs treated with different LPS concentrations.

LPS concentration (μg/mL)		*P* value	
		48 h	96 h
0	5	0.000*	0.000*
	10	0.000*	0.000*
	20	0.000*	0.001*
5	10	0.329	0.917
	20	0.937	0.229
10	20	0.000*	0.095

* Statistically significant difference compared to the baseline.

growth factor secreted by PDLSCs promotes angiogenesis and osteogenesis during periodontal reconstitution (Lee et al., 2012). However, the oral cavity harbours numerous bacterial toxins (even after conventional periodontal therapy), and they can impair stem cell activities during periodontal regeneration (Kittaka et al., 2013). The gradation design used in the current investigation shows the trend of cell proliferation and VEGF secretion of PDLSCs treated with a series of LPS concentrations.

Cell proliferation of PDLSCs decreased significantly in all LPS treated groups (5, 10 and 20 μg/mL of LPS) at both 48 and 96 h except for the group treated with 5 μg/mL of LPS at 48 h. This finding suggests that periodontal pathogens of dental plaque reduce the efficacy of PDLSCs during periodontal regeneration. This finding is in accordance with several previous studies which have investigated the effect of LPS on cell proliferation of various stem cells. Lan et al. (2013) demonstrated that 10 μg/mL of *P. gingivalis* LPS inhibited cell proliferation of PDLSCs, whereas Morsczeck et al. (2012) showed that 1 μg/mL of both *E. Coli* and *P. gingivalis* LPS inhibited the proliferation of DFCs. It has been shown that *P. gingivalis* LPS inhibits PDLSC proliferation directly or indirectly through LPS-induced inflammatory cytokines (Lan et al., 2013). In response to LPS, PDLSCs with their immunomodulatory properties produce pro-inflammatory cytokines mainly through the toll-like receptor 4 (TLR4) pathway that can influence cell proliferation of PDLSCs (Li et al., 2014; Zhu and Liang, 2015).

Interestingly, some previous studies documented the opposite results. Expansion of PDLSCs within the diseased periodontium was shown to have accelerated tissue healing (Cianci et al., 2016). Albiero et al. (2015) reported that PDLSCs treated with *E. coli* LPS remained proliferative at 100 ng/mL, 1 μg/mL and 10 μg/mL. Kato et al. (2014) showed that 1 to 10 μg/mL of *P. gingivalis* LPS promoted PDLSC proliferation. The findings of various studies have not been equivocal probably due to the different methodologies used. Therefore, the discrepancies could be attributed to various factors regulating the stem cell properties, for instance, tissue origin, donor age, inflammatory condition, culture method and growth factors (Zhu and Liang, 2015). In addition, the type of bacteria and LPS concentration used are important factors influencing the research results.

The LPS concentration of biofilm varies greatly depending on the local inflammatory microenvironment (Lin et al., 2008). Kato et al. (2014) revealed that PDLSCs treated with 0 to 10 μg/mL of *P. gingivalis* LPS showed the most pronounced proliferation at 10 μg/mL, whereas less than 1 μg/mL was not effective. However, the results of the current study showed there was no significant difference in cell proliferation of PDLSCs among the groups treated with different LPS concentration. Therefore, cell proliferation of PDLSCs was reduced independently of LPS concentration.

Also in this current study, cell proliferation of LPS treated PDLSCs showed no significant difference from 48 to 96 h. This finding suggests that PDLSCs remain viable over time and show a possible adaptive mechanism when subjected to continuous exposure to LPS. In a study done by Albiero et al. (2015), despite the presence of *E. coli* LPS (100 ng/mL, 1 μg/mL and 10 μg/mL), PDLSCs showed a time-dependent increase of proliferation for 10 days of culture, especially after day 3 and 7. The adaptive mechanism of PDLSCs works by downregulating the TLR4 gene expression that has been reported by numerous studies (Mo et al., 2008; Muthukuru et al., 2005). This property of endotoxin tolerance supports the potential use of PDLSCs for periodontal regeneration in diseased periodontium containing LPS which is not able to be removed completely by periodontal therapy (Kittaka et al., 2013). These concentrations of endotoxin are effectively sub-lethal for the PDLSCs.

In this study, VEGF secreted by PDLSCs was found to decrease statistically significantly in all LPS treated groups (5, 10, 20 μg/mL) at both 48 and 96 h. There was no statistically significant difference in VEGF concentration among the groups treated with varying LPS concentration. Therefore, the reduction in VEGF secretion by PDLSCs was independent of LPS concentration. One reason could be related to the decreased number of PDLSCs capable of secreting VEGF in the presence of LPS. However, this contrasts with a study done by Palaska et al. (2016) which reported that *P. gingivalis* and *E. coli* LPS induced a significant release of VEGF from mast cells. In addition, it could also be due to the effect of LPS on the gene expression of VEGF-A and -B mRNA of PDLSCs as the TLR4 regulated NF-kB pathway has been demonstrated to suppress the VEGF-A expression of infantile hemangioma-derived stem cells (Kittaka et al., 2013).

In the present study, prolonged exposure to LPS for 96 h was designed to mimic a situation in which PDLSCs interact

continuously with bacterial toxins in chronic periodontal disease. The present study observed a statistically significant increase in VEGF secretion from PDLSCs from 48 to 96 h regardless of the LPS concentration. This observation is supported by another finding in this study; PDLSCs remained viable over time in the presence of LPS and thus capable of secreting VEGF with time. Secondly, this may also be due to the adaptive mechanism of PDLSCs when subjected to the continuous exposure to LPS. VEGF increases with time to facilitate neovascularisation and osteogenesis necessary for periodontal repair (Mo et al., 2008).

As for the limitations of this study, it was an *in vitro* study with absence of other host cells such as immune cells which play an important role in the pathogenesis of the periodontal disease. The LPS toxin in this study setting was not replenished for the degeneration that could happen in the actual microenvironment and the lyophilized powder form could translate only to a certain extent. Therefore, more extensive studies are needed that mimic the actual periodontal microenvironment with a combination of various bacteria species and immune cells to investigate their effects on cell proliferation and VEGF secretion of PDLSCs.

To increase the therapeutic efficacy of periodontal regeneration, ways of modulating cell proliferation and paracrine action of PDLSCs under the influence of LPS should be investigated. The authors of this study suggest that VEGF supplement be used to compensate for the reduced VEGF production triggered by LPS during periodontal regeneration. Researches have been conducted on using growth factors in relatively high concentration to enhance the properties of scaffold materials and this approach has been proven successful (Ammar et al., 2018).

5. Conclusions

E. coli LPS reduces cell proliferation and VEGF secretion of PDLSCs, suggesting that periodontal pathogens reduce the efficacy of PDLSCs during periodontal regeneration. The decrease in cell proliferation and VEGF secretion is independent of the LPS concentration. It was also noted that LPS treated PDLSCs remain viable over time while VEGF increases significantly with time. This is suggestive of an adaptive mechanism of PDLSCs in response to LPS and it supports the application of PDLSCs in the diseased periodontium. Therefore, future research is needed to study the potential use of PDLSCs for periodontal regeneration.

Ethical statement

There was no animal experiment carried out for this article.

Declaration of Competing Interest

No potential conflict of interest relevant to this article was reported.

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