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ORIGINAL ARTICLE

TERM

The Effects of Platelet-Rich Plasma on the Proliferation and Release of Growth Factors from Periodontal Ligament Cells

Gyu-Un Jung[1], Sun-Kyoung Lee[2], Suk Ji[3], Eun-Kyoung Pang[1,2]*

[1]Department of Periodontology, Ewha Womans University Mokdong Hospital, Seoul, Korea
[2]Department of Periodontology, School of Medicine, Ewha Womans University, Seoul, Korea
[3]Department of Periodontology, Korea University Anam Hospital, Seoul, Korea

Periodontal ligament cells (PDLCs) play an important role in the regeneration of periodontium. The healing potential of PDLCs may be due to the high concentration of growth factors in platelet-rich plasma (PRP). In this study, the effects of PRP on the proliferation and activation of PDLCs and growth factor release from PDLCs were investigated. PDLCs were isolated from third molars or premolars of healthy patients. Whole blood was obtained from healthy volunteers for the preparation of activated PRP. The platelet concentration in PRP was measured and the amount of platelet-derived growth factor (PDGF)-AB, PDGF-BB, transforming growth factor-β1 (TGF-β1), and vascular endothelial growth factor (VEGF) were determined by enzyme-linked immunosorbent assay. The effects of activated PRP on PDLC proliferation, attachment, alkaline phosphatase (ALP) activity, and growth factor release were investigated. Platelet concentration was increased 5.41-fold in PRP compared to whole blood. PDGF-AB, PDGF-BB, TGF-β1, and VEGF were detected in PRP at concentrations of 273.38 ng/mL, 47.0 ng/mL, 168.42 ng/mL, and 510.56 pg/mL, respectively. PDLCs cultured with ≥10% PRP showed significantly increased cell proliferation and ALP activity compared to the control (p<0.05). PDLCs cultured with 10% PRP also presented higher cell attachment and increased release of TGF-β1 and VEGF compared to the control (p<0.05). PRP can deliver high concentrations of growth factors to a defect site to increase the proliferation, attachment, and ALP activity of PDLCs. These results suggest that PRP might effectively contribute to periodontal regeneration.

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Key Words: Periodontal ligament; Regeneration; Platelet-rich plasma; Growth factor; Vascular endothelial growth factor

INTRODUCTION

The principal goal of periodontal tissue regeneration is restoration of dental cementum, periodontal ligament, and alveolar bone that is lost due to periodontal disease [1]. Epithelial cells, gingival fibroblasts, periodontal ligament cells (PDLCs), osteoblasts, and other cell types participate in regeneration of periodontium. PDLCs play a particularly essential role; thus, many attempts have been made to induce healing by stimulating PDLCs.

Classically, guided tissue regeneration methods using resorbable or non-resorbable membranes have been used to inhibit the migration of epithelial cells and to stimulate PDLCs. Recently, methods using growth factors and enamel matrix derivatives have been introduced. Of these, growth factors are essential biologic mediators that effect cell proliferation, differentiation, and synthesis of extracellular matrix. One of the methods that can effectively deliver high concentrations of growth factors to wounds is platelet-rich plasma (PRP). PRP is produced by concentrating platelets from whole blood and contains large amounts of growth factors released from platelets. α-Granules of platelets contain platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) and when activated, release these growth factors to promote wound healing [2,3]. PRP has been used in orthopedics, plastic surgery, oral maxillofacial surgery, dental implant surgery, and recently, periodontal surgery [4-8].

Gruber et al. [9,10] reported that PRP stimulates DNA synthesis in osteoblasts and increases migration and proliferation of

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***Corresponding author:** Eun-Kyoung Pang, Department of Periodontology, School of Medicine, Ewha Womans University, 1071 Anyangcheon-ro, Yangcheon-gu, Seoul 07985, Korea.
Tel: 82-2-2650-2725, Fax: 82-2-2650-5764, E-mail: ekpang@ewha.ac.kr

bone marrow mesenchymal stem cells. Also, Liu et al. [11] reported that PRP increases the proliferation of fibroblasts. Similarly, many studies have reported the effects of PRP on fibroblasts, osteoblasts, mesenchymal stem cells, and other cell types [11]. However, information related to the effect of PDLCs is limited, and there are no data regarding the mechanism by which PRP induces the release of growth factors from PDLCs. Thus, the aim of this study was to investigate the effects of PRP on proliferation, alkaline phosphatase (ALP) activity, cell attachment, and release of growth factors from PDLCs *in vitro*.

MATERIALS AND METHODS

All procedures followed were in accordance with the ethical standards of the Ewha Womans University Mokdong Hospital Institutional Review Board and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

Materials

Cell isolation and culture

PDLCs were prepared from extracted premolars that were removed for orthodontic reasons or extracted third molars from 10 young systemically and periodontally healthy volunteers (six males and four females, aged 25 to 30 years). The periodontal ligament tissues were obtained from the middle one-third of roots, minced, and placed in 60-mm culture dishes in Dulbecco's modified minimum essential medium F-12 (DMEM/F12; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/mL penicillin, 100 μg/mL streptomycin, and overlaid with coverslips. After culturing the cells in DMEM, they were rinsed in phosphate-buffered saline to remove unattached cells, then they were incubated in 95% air, 5% CO_2, 100% relative humidity, and 37°C. Culture medium was replaced once every two days. Outgrowing cells were harvested using trypsin-ethylene diamine tetraacetic acid (GIBCO) when the culture had reached 80% confluence and subcultured in fresh culture medium. Cells of the fourth to sixth passages were used for the experiments described below.

Preparation and activation of PRP

Whole blood was obtained from three systemically healthy adults (two males and one females), 25–30 years old, and stored in tubes with anticoagulant citrate dextrose (Sigma-Aldrich, St. Louis, MO, USA). PRP was prepared using a two-step centrifugal separator specially made for PRP preparation (PLACON, Oscotec, Seoul, Korea) following the manufacturer's instructions. In brief, whole blood was initially centrifuged at 2000×g for 3 min and plasma was decanted up to the red blood cell sediment. Then, plasma was again centrifuged at 5000×g for 5 min and platelet-poor plasma on the top was removed. Then, 1 mL of PRP per 10 mL of whole blood was obtained at the bottom. The platelet levels of whole blood and PRP were counted using a Coulter STKS platelet counting machine (Beckman-Coulter, Chicago, IL, USA). Prepared PRP was activated by combining of 14.3 mg/mL $CaCl_2 \cdot 2H_2O$ (Calmia; Oscotec) and 142.8 U/mL thrombin (Thirabin; Oscotec) at 37°C for 1 h. The mixture was centrifuged at 4000×g for 10 min at 4°C and the supernatant was stored at -70°C until used.

Methods

Quantitative analysis of growth factors in PRP

The PDGF-AB, PDGF-BB, TGF-β1, and VEGF levels of PRP were determined by enzyme-linked immunosorbent assay (ELISA; Duoset, R&D Systems, Inc., Minneapolis, MN, USA).

Effects of different PRP concentrations on PDLC proliferation and ALP activity

The PDLCs were seeded in 96-well plates at 1×10^4 cells/well and incubated in 100 μL DMEM containing 10% FBS. After culturing the cells in DMEM, they were rinsed in phosphate-buffered saline to remove unattached cells, then incubated further in DMEM. After 24 h, the medium was replaced with serum-free DMEM. After 24 h, the medium was replaced with DMEM containing PRP in the test group, and serum-free DMEM in the control group. Five subgroups were set in the PRP group by concentrations of 0, 2.5, 10, 20, and 40% PRP. After 3 days of incubation, the following analyses were performed.

Cell proliferation assay

Cell proliferation analysis was performed using a cell proliferation assay kit [Cell Counting Kit-8 (CCK-8), Donjindo Lab., Kumamoto, Japan] following the manufacturer's instructions. The media of the test and control groups were replaced with 100 μL serum-free DMEM with 10 μL CCK-8 solution. This cell viability assay is based on the enzymatic reduction of tetrazolium salt to formazan dye, the amount of which is directly proportional to the number of viable cells. After 4 h of incubation, absorbance was determined at 450 nm with a microplate reader.

ALP activity analysis

ALP activity in the cell layer was determined using a QuantiChrom Alkaline Phosphatase Assay Kit (BioAssay Sys., Hayward, CA, USA). The cells of the test and control groups were washed twice with Dulbecco's phosphate-buffered saline. Fifty microliters of 0.2% Triton X-100 was added and the tissue cul-

ture plates were shaken for 20 min. ALP analyzing reagent (150 μL of 1 M p-nitrophenyl phosphate/0.2 M Mg acetate) was added and incubated at 37°C for 30 min. ALP catalyzes the hydrolysis of p-nitrophenyl phosphate into inorganic phosphate and p-nitrophenol, which is yellow. After stopping the reaction by adding 100 μL of 1 M NaOH, p-nitrophenol was measured at 405 nm using the microplate reader.

PRP concentration on PDLC attachment

10 μL PRP or serum-free DMEM medium was added to each well of a 96-well plate and in the test and control groups, respectively. PDLC density was adjusted to 2×10^4 cells/mL and 90 μL was added to each well. After incubating for 4 h under standard incubation conditions, cell viability was assessed by a cell proliferation assay as described above. The amount of color produced was directly proportional to the number of attached viable cells, while non-attached cells had been washed off.

Release of PDLC growth factors

PDLCs (3×10^5) were seeded in 35-mm culture dishes and incubated in DMEM supplemented with 10% FBS. After 24 h, culture medium was replaced with serum-free DMEM. After 24 h, the test group was incubated in DMEM medium supplemented with 10% PRP and the control group was incubated in serum-free DMEM medium. The conditioned media were harvested after 0, 8, 24, 48, 72, and 96 h. Then, the level of PDGF-AB, PDGF-BB, TGF-β1, and VEGF in conditioned media was measured by ELISA.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). In the cell proliferation and ALP activity assays, statistical analysis was performed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. In measurements of cell attachment level, the Student's t-test was performed. The effect of PRP on the release of growth factors from PDLCs over time was analyzed by two-way ANOVA and Bonferroni's multiple comparison test. For all tests, statistical significance was established as $p<0.05$.

RESULTS

Quantitative analysis of growth factors in PRP

Platelet counts in the PRP preparations were 11.10×10^8 cells/mL. In contrast, platelet counts in non-concentrated whole blood were 2.05×10^8 cells/mL. Thus, the concentration of platelets in PRP was increased 5.41-fold (Table 1). The supernatant of activated PRP contained 273.38 ng/mL PDGF-AB, 47.0 ng/mL PDGF-BB, and 168.42 ng/mL TGF-β1 (Table 2). The level of VEGF was 510.56 pg/mL and lower than that of PDGF and TGF-β1.

Effects of different PRP concentrations on PDLC proliferation and ALP activity

PDLC proliferation showed a statistically significant increase in the test group, which contained PRP in culture medium, compared to control ($p<0.05$). PDLC proliferation dose-dependently increased in 0 to 20% PRP, and then showed a statistically significant decrease in 40% PRP ($p<0.05$). There was no statistically significant difference between the 10 and 20% PRP groups (Fig. 1). The ALP activity of PDLCs incubated at different PRP concentrations for 72 h was measured. There were statistically significant differences in the 10, 20, and 40% PRP gr-

Table 1. Platelet counts in whole blood and PRP

Patient	Whole blood (cells×10^8/mL)	PRP (cells×10^8/mL)	PRP/whole blood ratio (fold)
A	2.33	12.10	4.42
B	1.98	10.31	6.11
C	1.84	10.89	5.91
Mean±SD	2.05±0.25	11.10±0.91	5.41±0.92

PRP: platelet-rich plasma, SD: standard deviation

Table 2. Growth factor levels in PRP

Patient	PDGF-AB (ng/mL)	PDGF-BB (ng/mL)	TGF-β1 (ng/mL)	VEGF (pg/mL)
A	302.58	58.35	182.28	531.49
B	255.42	39.48	155.65	498.01
C	262.14	43.17	167.33	502.18
Mean±SD	273.38±25.51	47.0±10.00	168.42±13.34	510.56±18.24

PRP: platelet-rich plasma, PDGF: platelet-derived growth factor, TGF-β1: transforming growth factor-β1, VEGF: vascular endothelial growth factor, SD: standard deviation

oups compared to the control (p<0.05). The test group incubated with 2.5% PRP showed no statistically significant difference compared to the control. ALP activity increased dose dependently in the 10, 20, and 40% PRP groups; however, there were no statistically significant differences between these groups (Fig. 2).

Effect of PRP on PDLC attachment and growth factor release

Considering these results, 10% PRP was deemed an appropriate concentration that showed statistically significant effects compared to the control. Thus, the following experiments were performed with 10% PRP in the test group. The 10% PRP group showed a significant increase in PDLC attachment (1.2-fold; p<0.05), compared to the control (Fig. 3). In the test group, there were two sources of growth factors: PRP and PDLCs. To calculate the level of growth factors released from PDLCs, the growth factor levels of 10% PRP were subtracted from the test group. Thus, the following results were analyzed based on the net gr-

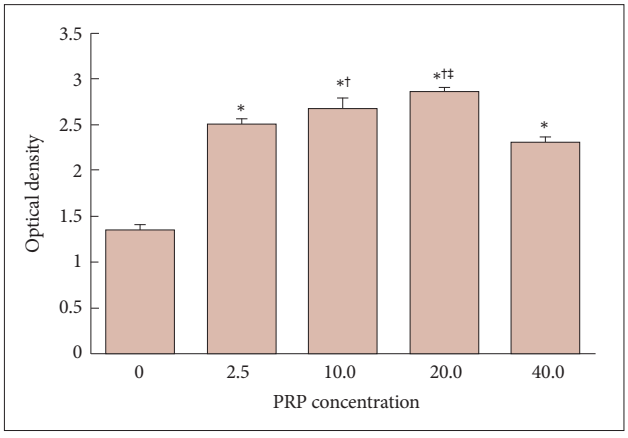

Figure 1. The effect of different platelet-rich plasma (PRP) concentrations on the proliferation of periodontal ligament cells. The results represent the means±standard deviation of three replicates. *Statistically significant difference from 0% PRP group (p<0.05), †Statistically significant difference from 40% PRP group (p<0.05), ‡Statistically significant difference from 2.5% PRP group (p<0.05).

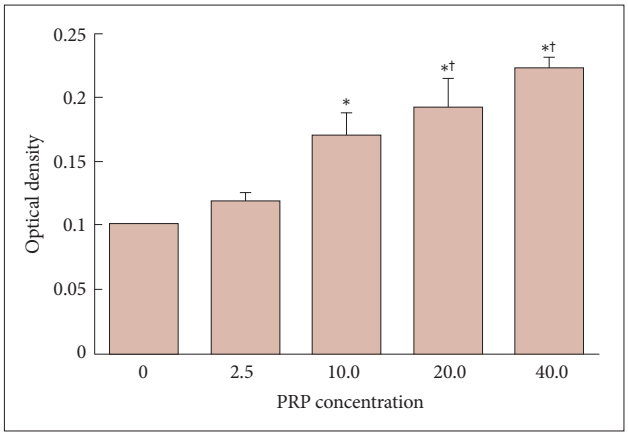

Figure 2. The effect of different platelet-rich plasma (PRP) concentrations on the alkaline phosphatase activity of periodontal ligament cells. The results represent the means±standard deviation of three replicates. *Statistically significant compared to 0% PRP (control) group (p<0.05), †Statistically significant difference from 2.5% PRP group (p<0.05).

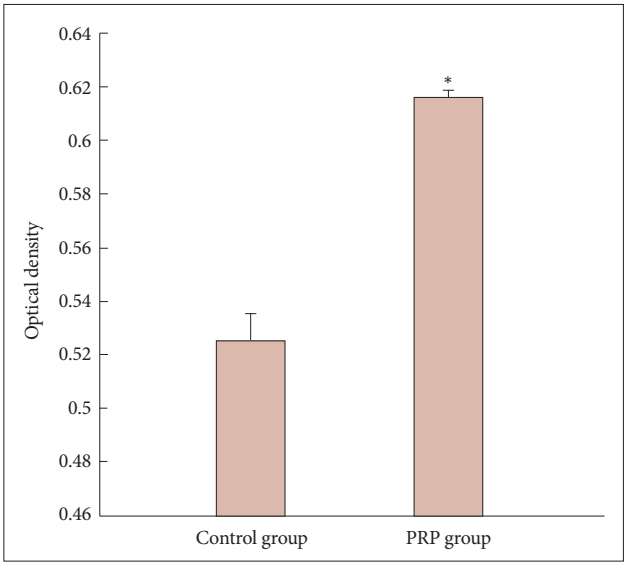

Figure 3. The effect of platelet-rich plasma (PRP) on the attachment of periodontal ligament cells. The results represent the means± standard deviation of three replicates. Control group, serum-free Dulbecco's modified minimum essential medium (DMEM); PRP group, 10% platelet-rich plasma in serum-free DMEM. *Statistically significant compared to control group (p<0.05).

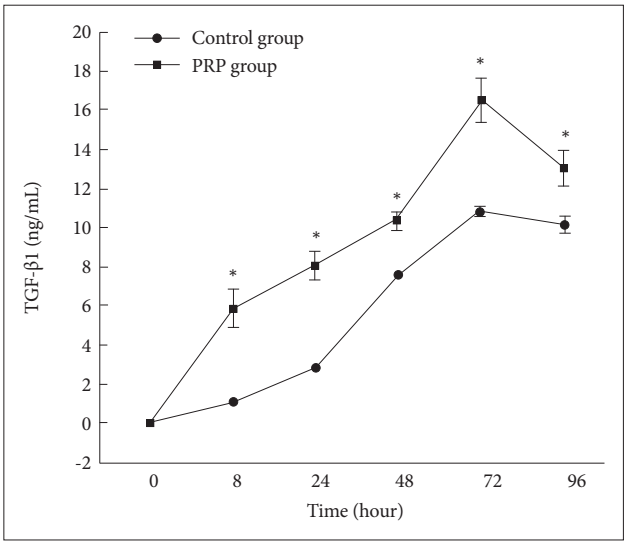

Figure 4. Time-dependent effect of platelet-rich plasma (PRP) on the release of transforming growth factor-β1 (TGF-β1) from periodontal ligament cells. The results represent the means±standard deviation of three replicates. Control group, serum-free Dulbecco's modified minimum essential medium (DMEM); PRP group, 10% platelet-rich plasma in serum-free DMEM. *Statistically significant compared to control group (p<0.05).

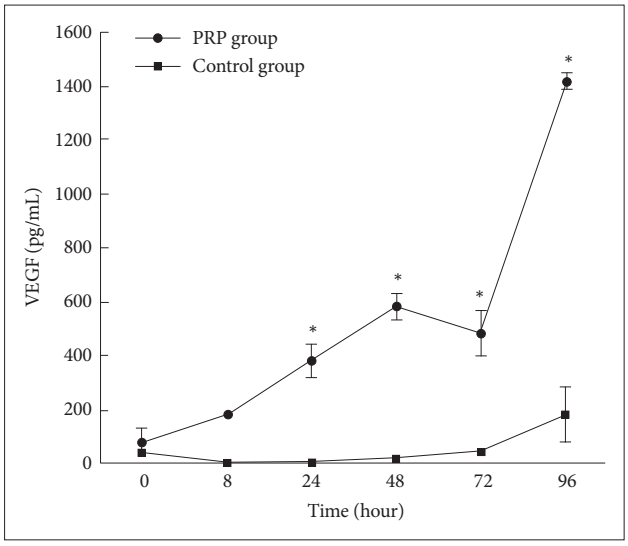

Figure 5. Time-dependent effect of platelet-rich plasma (PRP) on the release of vascular endothelial growth factor (VEGF) from periodontal ligament cells. The results represent the means±standard deviation of three replicates. Control group, serum-free Dulbecco's modified minimum essential medium (DMEM); PRP group, 10% platelet-rich plasma in serum-free DMEM. *Statistically significant compared to control group (p<0.05).

owth factor levels of PDLCs only. When PDGF-AB and PDGF-BB levels were measured time-dependently in the 10% PRP and control groups, PDGF-AB and PDGF-BB were not detected in the control group. In addition, in the 10% PRP group, PDGF-AB was not detected at 24 h and PDGF-BB was not detected at 8 h. Thus, these data were excluded from this study.

TGF-β1 levels significantly increased from 24 h in the control and 8 h in the test group, and the peak was 72 h (p<0.05). At 96 h, there was a decrease in both the control and test groups, but this was not statistically significant. The TGF-β1 level of the test group was higher than that of the control at 8 h, and this difference continued to 96 h (p<0.05) (Fig. 4).

In the control group, VEGF was not detected at 8 or 24 h, but was detected at 48 h and beyond. The peak was at 96 h (179.63 pg/mL), but there were no significant differences throughout. In the test group, the VEGF level significantly increased from 24 h and largely increased from 72 h to 96 h, and peaked at 96 h (p<0.05) (Fig. 5).

DISCUSSION

Reports on the effectiveness of PRP have been published, and several studies have reported an increase in bone formation and growth, while a contrasting report suggested that PRP has no adjunctive effect [3,12-20]. Furthermore, controversies about the clinical usefulness of PRP persist [21-23]. In the present study, the effect of PRP on PDLCs, which are important in periodontal tissue regeneration, was investigated and the utility of PRP in modulating cytological levels was determined.

As a result, PRP produced a 5.41-fold increase in platelets compared to whole blood. The methods for producing PRP are variable, and platelet counts differ from person to person; thus, reported platelet concentrations vary widely. Studies have reported increases in platelet concentrations from 2.05 to 8.73-fold vs whole blood [3,24].

PDGF-AB was the most highly concentrated growth factor in PRP, followed by TGF-β, PDGF-BB, and VEGF. The VEGF level was much lower than that of other growth factors and was less than that of PDGF-BB. Okuda et al. [12] reported 182.0 ng/mL PDGF-AB and 140.9 ng/mL TGF-β1 in PRP, and Roussy et al. [25] reported 50.4 ng/mL TGF-β1, 15.0 ng/mL PDGF-BB, and 250 pg/mL VEGF. Thus, the growth factor levels in the present study were higher than those reported in the other studies. PRP measurements also have limitations in that platelet concentration is unpredictable and growth factor levels cannot be controlled. Therefore, some authors prefer recombinant human PDGF (rhPDGF) and TGF-β (rhTGF-β) [26,27]. However, Han et al. [28] pointed out that application of PRP can increase the growth of PDLCs and ALP activity more than rhPDGF and rhTGF-β because PRP has a greater variety of growth factors, including IGF and EGF and many factors related to healing.

In the present study, our experiments using different concentrations of PRP showed that cell proliferation was increased about 1.7−2.1-fold over that of the control. PRP at 20% showed the highest cell proliferation rate, although there were no statistically significant differences in other PRP concentrations. Intriguingly, the cell proliferation rate at 40% PRP was lower than that at 20%. In a report by Graziani et al., [29] which investigated the effects of PRP on gingival fibroblasts and osteoblasts, the authors mentioned that the highest concentration of PRP did not always show the highest cell proliferation rate. Also, Liu et al. [11] compared 8.8, 17.5, and 35% PRP and revealed that 8.8 and 17.5% showed more proliferation of fibroblasts than 35%, and postulated that highly concentrated PRP changes the pH of the media, thereby inhibiting proliferation.

ALP activity, an indicator of early osteoblastic differentiation, was assayed to examine the effect of PRP on the bone-formation capacity of PDLCs. Our results show that PRP induced higher ALP activity than the control. The increase in activity was about 1.7- to 2.2-fold (in 10% and 40% PRP, respectively, and these values are consistent with the increase in cell proliferation rate as in optical density. Therefore, the increase in ALP activity in the test group can be assumed to be an indirect effect of increased PDLC number, not a direct effect of PRP itself. With regard to the cell proliferation and ALP activity assays, 10% PRP was thought to be an appropriate concentration that

showed statistically significant effects compared to control. Epithelial cells can attach more quickly to root surfaces than PDLCs and therefore present difficulties in achieving ideal periodontal tissue regeneration [30,31]. Okuda et al. [12] reported that PRP increased the number of PDLCs and decreased the number of epithelial cells. Therefore, use of PRP can inhibit epithelial cells, stimulate PDLC proliferation, and increase PDLC attachment to root surfaces and bone walls of defect areas. Taken together, these results suggest that PRP can effectively contribute to periodontal tissue regeneration.

To analyze the effects of PRP on the levels of growth factors released from PDLCs, the growth factor levels of 10% PRP were subtracted from those of the test group. Although PDGF-AB and PDGF-BB were the most abundant growth factors in PRP, these factors were not released from PDLCs at 24 or 8 h, respectively, in the test group. The PDGF could act as a chemotactic agent in recruiting preosteoblast to ths site of bone injury area, and also stimulate mitogens of osteoblastic precursors [24]. Similarly, other studies have been suggested that application of PDGF to treat periodontal defects revealed the partial regeneration of periodontal ligament, cementum, and alveolar bone *in vivo* studies [32-35]. Therefore, the stimulating effect of PDGF-AB and PDGF-BB on periodontal tissue regeneration is presumed to act in the early healing phase and is attributed to PRP itself, not to PDLCs.

The most abundant growth factor secreted by PDLCs was TGF-β1. In both the control and test groups, TGF-β1 levels increased over time and peaked at 72 h, and slightly decreased at 96 h. The gap between the test and control groups was observed after 8 h and the statistically significant differences were maintained after that. In a previous studies of TGF-β1, it has been shown to stimulate the proliferation and synthesis of collagen by osteoblast and its precursor [36-38].

VEGF was rarely detected in the control group. However, VEGF in the 10% PRP group was significantly detected after 24 h and steeply increased from 72 h to 96 h. This observation suggests that PRP can stimulate the production and/or secretion of VEGF from PDLCs, which do not typically release VEGF. VEGF is a major contributing factor in angiogenesis; as little as 100–150 pg/mL VEGF can produce a half-maximal effect in vascular endothelial cells *in vitro*, and 1–4 ng/mL shows a maximal effect in endothelial cell proliferation [39]. In the present study, the combined VEGF level, i.e., that secreted from PDLCs at 96 h plus that in PRP, reached an average value of 690.2 pg/mL.

Several previous studies have identified that cell growth, attachment, and proliferations play an important role in periodontal tissue regeneration [32-38]. In this study, PRP stimulated increase growth factors from PDLCs and provided benefits for periodontal regeneration.

Conclusions

In summary, PRP contains various growth factors including PDGF-AB, PDGF-BB, TGF-β1, and VEGF. We show here that PRP stimulates the release of TGF-β1 and VEGF from PDLCs. Thus, PRP can deliver abundant growth factors to defect areas both directly and indirectly to increase PDLC proliferation, attachment, and ALP activity. These functions can effectively contribute to periodontal regeneration in defect areas. Finally, PRP is considered an effective tool for facilitating periodontal regeneration. Future *in vivo* studies will be needed to clarify the mechanistic details and potential clinical utility of PRP-mediated periodontal regeneration.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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