Freeze-Dried human platelet-rich plasma
effect on osteoblast number and collagen
density in periodontitis treatment

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Abstract

Objective: This study aimed to determine the effect of FD-hPRP on the number of osteoblast and collagen density in periodontitis treatment by a histomorphometric study on oryctolagus cuniculus rabbits' alveolar bone and periodontal ligament.

Material and Methods: Sixteen male rabbits were randomly divided into four groups: Normal, Periodontitis, FD-hPRP, DFDBA. After periodontal surgery by open flap debridement, FD-hPRP was applied in group 3, and DFDBA was applied in group 4. Then all rabbits were decapitated eight weeks after surgery. Histomorphometric analysis calculates the number of osteoblasts and collagen density using image J software. Data were analyzed using one-way ANOVA followed by Post Hoc Least Significant Difference (LSD) test.

Results: The results showed a significant difference (p<0.05) between the periodontitis and FD-hPRP and also DFDBA group.

Conclusion: The application of FD-hPRP effect increases the number of osteoblast and collagen density in periodontitis treatment histomorphometrically in rabbit's alveolar bone and periodontal ligament.

Keywords: Bone graft, Freeze-drying, Human platelet-rich plasma, Periodontal regenerative
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Introduction

Periodontitis is a periodontal disease triggered by an immune response to a bacterial cause progressive damage to the periodontal ligament and alveolar bone.1 The main goal treatment of periodontal treatment has attracted researchers' interest in treatments for regenerating periodontal tissue and forming new bone.5,6 The ordinarily used periodontal tissue treatment therapy is open flap debridement (OFD), but it has limitations in achieving the desired regeneration results.5 The ability of tissue engineering approaches based on bone graft materials and biologic mediators with human platelet-rich plasma (hPRP) is the reason for achieving bone healing, restoring attachment to the periodontal ligament, and supporting OFD treatment in regenerating periodontal treatment tissue.5,6

Demineralized freeze-dried bone Allograft (DFDBA) is an allograft bone graft consisting of a demineralized bone matrix derived from cadavers declassified in hydrocolloid acid, which can regenerate periodontal tissue.7 The advantages of DFDBA are biocompatibility, no allergies, and osteoinduction properties by inducing mesenchymal cells to become osteoblasts. However, its use still has several weaknesses, including its osteoinduction ability which depends on the age of the bone donor and its scarce availability.3

The use of human platelet-rich plasma (hPRP) in tissue engineering can provide significant changes to tissue healing as an alternative to natural bone graft materials since autologous is not always the best solution because it has enormous variability results.9 Human PRP (hPRP) or homologous PRP is a carrier of growth factors taken from the product not used in the Indonesian Red Cross (PMI). Human PRP contains growth factors that benefit vascularity, proliferation and maturation of osteoblasts and fibroblasts. The problems often encountered in clinical applications are the short storage time of PRP, the liquid nature to dissolve quickly, and patients must take a large amount of blood before surgery to prepare new PRP.10

The freeze-drying method is a consistent standardization method to maintain the stability of hPRP to obtain hPRP in ready-to-use powder form and increase Transforming growth factor-beta (TGF-β) levels and platelet-derived growth factors (PDGF) contained in hPRP.10-12 Furthermore, the safety of FD-hPRP is predominant and demonstrated in pilot studies regarding compatibility before donation,13 and sterilization is needed to enhance the bioactivity and storage of FD PRP for a long time, and that sterility can be achieved by 25 kGy gamma irradiation without affecting platelets in osteoblast behavior (migra-
Table 1. Result of data showing mean± standard deviation (SD) values for osteoblast cells number in four study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD-hPRP</td>
<td>4</td>
<td>29.18 ± 0.80</td>
<td>0.355</td>
</tr>
<tr>
<td>DFDBA</td>
<td>4</td>
<td>28.69 ± 0.56</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference between groups (p<0.05)

Table 2. Result of data showing mean± standard deviation (SD) values for osteoblast cells number in four study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontitis</td>
<td>4</td>
<td>0.00 ± 0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Health</td>
<td>4</td>
<td>60.90 ± 1.15</td>
<td></td>
</tr>
<tr>
<td>FD-hPRP</td>
<td>4</td>
<td>59.83 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>DFDBA</td>
<td>4</td>
<td>59.20 ± 0.63</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference between groups (p<0.05)

Figure 1. Histological view of osteoblast (red arrow) on the alveolar bone of Oryctolagus cuniculus with HE staining: A. Healthy group, B. Periodontitis group, C. FD-hPRP group, D. DFDBA group.

Material and Methods

Preparation of freeze-dried human platelet-rich plasma

This research was an experimental animal and was approved by Fakultas Kedokteran Hewan Universitas Gadjah Mada ethical committee with the Certificate of Ethical Eligibility number 0025/EC-FKH/Ex./2020. Human PRP was obtained from the blood bank of PMI. The whole blood was centrifuged (RC KUBOTA 9942) at 32XG in 4 for 30 minutes to separate platelet-rich plasma (PRP) and the red blood cell (RBC) fraction. An average of 100-120mL PRP and obtained from the O blood group at the end of this procedure. Human PRP was frozen at the temperature of -40 for 12h. This freeze-dried process was then continued using a freeze-drier machine for 48h (Freeze Dryer Modulo, Edwards). Lastly, these freeze-dried were mashed with a mortar and filtered using a 60-mesh filter inside the laminar flow hood station and sterilization 25k Gy by gamma irradiation.

Experimental Animal Procedure

Sixteen male rabbits (oryctolagus cuniculus) used 5-8 months old weighing 1500–2000 g were induced by periodontitis. Periodontitis induction in rabbits were performed by ligation technique and lipopolysaccharide injection (LPS) from porphyromonas gingivalis ATCC 33277 from Thermo Scientific, USA. First, rabbits were acclimatized for one week. Four rabbits as the health group were randomly selected to be decapitated. Induction of periodontitis was then performed under anesthesia using ketamine HCL 40 mg/kg body weight (BW) and xylazine 3 mg/kg by intramuscular injection. The cervical area of the mandibular anterior teeth was tied using silk 3.0 for six weeks. 0.05 ml LPS from porphyromonas gingivalis was injected three times a week into the area of the mandibular anterior teeth.18 Rabbits as periodontitis model animals were randomly divided into four groups. The clinical signs observed in the periodontitis rabbit were tooth mobility, gingival recession, and gingival redness. Furthermore, after periodontitis, four rabbits were randomly selected to be decapitated as the periodontitis group.

Surgical protocol

Open flap debridement was performed under anesthesia using ketamine 40mg/kg BW and xylazine 5mg/kg BW. Sulcular incision using a scalpel no.15 in the area of the buccal groove of the mandibular central incisors, and then reflected using a small raspatorium. The soft and hard tissues were debrided, then irrigated with distilled water and repositioned the flap by sutur-
increased PDGF levels, which play a role in recruiting immune cells such as T cells. PDGF plays a role in the wound healing process (inflammation, proliferation, and regeneration) and plays a role in the wound healing process. TGF-β is very important for soft tissue regeneration. It is in line with growth factors that play a role in each phase of periodontal tissue regeneration.

Results

Osteoblast Number
During the postoperative period, no animal presented complications or infection in the operated region. The grafted biomaterials were not exposed to any animal during the healing period until their euthanasia. Figure 1 shows the number of osteoblast cells in HE staining for the health group figure 1A, the periodontitis group figure 1B, the FD-hPRP group figure 1C, and the DFDBA group figure 1D. Osteoblast cells in the health, FD-hPRP and DFDBA group were seen on the edges of the bony trabeculae marked with yellow arrows. Osteoblast cells are cuboidal in shape with a brownish cell nucleus. Defect area in the alveolar bone contained osteoblasts for all treatment groups, indicating ongoing bone formation. Meanwhile, in the periodontitis group, there were no osteoblast cells. The entire defect area in the alveolar bone is filled with inflammatory cells shown in bright red, round shape, and spread. It is shown that the osteoclastogenesis process is taking place figure 1B.

Table 1 reported the result of histologic data osteoblast number. The highest number of osteoblasts was observed in the health group (30.00±1.00), while the lowest number was observed in the periodontitis group (0.00±0.00). The Shapiro-Wilk normality test showed that the osteoblasts number in each treatment group was normally distributed with p>0.05. The Levene homogeneity test showed that all data were homogeneous with p>0.05. A one-way ANOVA parametric statistical test was performed based on the normality and homogeneity test results. The difference between periodontitis groups and the health group, the FD-hPRP group and the DFDBA group, was statistically significant (ANOVA with post hoc LSD test;p<0.05); the difference between the FD-hPRP group and DFDBA group was not statistically significant (ANOVA with post hoc LSD test;p>0.05). Data shows that the treatment given affects osteoblast cells number.

Percentage of Collagen Density

Figure 2 shows the presentation of collagen density in the Mansson Trichrome stain for the health group figure 2A, the periodontitis group figure 2B, the FD-hPRP group figure 2C, and the DFDBA group figure 2D. Collagen density pictures in the FD-hPRP and DFDBA groups show green areas on the periodontal ligament indicating periodontal tissue regeneration. Meanwhile, in the periodontitis group, there were no green areas. The entire defect area of the peri-
The result of collagen density is summarized in Table 2. The highest percentage of collagen density was observed in the health group (60.90±1.15), while the lowest number was observed in the periodontitis group (0.00±0.00). The Shapiro-Wilk normality test showed that collagen density in each treatment group was normally distributed with p>0.05. The Levene homogeneity test showed that all data were homogeneous with p>0.05. A one-way ANOVA parametric statistical test was performed based on the normality and homogeneity test results. The difference between periodontitis groups and the health group, the FD-hPRP group and the DFDBA group, was statistically significant (ANOVA with post hoc LSD test; p<0.05); the difference between the FD-hPRP group and DFDBA group was not statistically significant (ANOVA with post hoc LSD test; p>0.05). The data show that the treatment given influences the percentage of collagen density.

Discussion

The periodontitis group showed the absence of osteoblasts and collagen density. It is due to the treatment of periodontitis induction by injecting LPS bacteria Porphyromonas gingivalis and ligation of the incisors mandibular rabbit can trigger the release of pro-inflammatory mediators such as IL-1, IL-6, and TNF-α. This inflammatory response increases the expression of matrix metalloproteinases (MMPs), leading to a decrease in collagen and establishing osteoclasts. The FD-hPRP and the DFDBA group show that applying the FD-hPRP and DFDBA material to the defect areas that OFD has done can increase osteoblast number close to osteoblast of the health group normal condition.

It is similar to the density of collagen in the periodontal ligament. The alveolar bone and periodontal tissue regeneration occurred in the two treatment groups. These results align with the study of Olivia et al. where FD-hPRP can induce osteoblast migration in vitro compared to the control group. The regeneration process occurs because FD-hPRP has osteoinduction potential that comes from growth factors, including TGF-β. The freeze-drying process in FD-hPRP can increase TGF-β levels. It is in line with Markopoulou et al. research regarding the effect of hPRP on osteoblasts in human periodontal tissue. The results showed that in vitro hPRP could increase the proliferation of osteoblasts in the periodontal tissues of patients with periodontitis aggressively so that hPRP could be used to treat bone defects in periodontal tissues.

Bone regeneration is a process that involves cellular signaling pathways that are triggered by various growth factors and biomolecules. The study of Berendsen and Olsen showed that TGF-β was associated with bone healing and increased bone regeneration. In intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts. The development of mesenchymal cells with osteogenic lineages that differentiate into osteogenic cells is controlled by the runt transcription factor (Runx2), the main transcription factor of osteoblastogenesis. Runx2 promotes the differentiation of mesenchymal cells into preosteoblasts and gene expression during the early stages of osteoblast differentiation. Osteoblast differentiation and maturation assisted by the transcription factor osterix (Osx) can trigger an increase in alkaline phosphatase (ALP) activity and mineralization.

The increase of TGF-β can trigger the inflammatory phase, thereby increasing the proliferation of the initial differentiation of progenitor and mesenchymal cells. TGF-β signaling is reliable with other signaling pathways to differentiate osteoblasts, signaling cross-talk between PDGF growth factors, Vascular Endothelial Growth Factor (VEGF) specifying several different growth factors that play an essential role in bone regeneration. TGF-β signaling can increase proliferation, chemotaxis, and early differentiation of osteoprogenitor, collagen synthesis, mineralization, and migration to osteocytes. In bone healing, the PDGF cannot work alone. PDGF synergizes with TGF-β on signaling cross talk in osteogenic differentiation. The expression of ALP is a temporary early marker of osteogenic differentiation in the cell mesenchyme, ALP expression at the time of osteogenic differentiation induced by TGF-β during the early stages mesenchymal cell osteogenesis will increase with the support of PDGF. TGF-β and BMP have different functions in osteogenesis, including mesenchymal condensation and osteoblast differentiation. BMP is a slow-acting growth factor after the initial inflammatory healing process is complete. BMP-2 and -6 exercise osteoblast differentiation, whereas BMP-4 and -7 moderately stimulate osteoblasts by increasing ALP expression and activity in early osteoblast progenitors.

The tissue regeneration process occurs because FD-hPRP has the osteoinduction potential from growth factors. The PDGF, TGF-β, VEGF, Insulin-like Growth Factor (IGF), Endothelial-like Growth Factor (EGF), and basic...
fibroblast growth factor (bFGF), including growth factors that play a role in each phase of soft tissue regeneration.\textsuperscript{25,26} It is in line with Creeper and Ivanovski’s\textsuperscript{27} study, which showed that the in-vitro effect of hPRP on fibroblasts in human periodontal tissue suggests that the use of hPRP improves fibroblast function related to wound healing and has the potential to result in faster tissue healing. This growth factor initiates release 10 minutes after the formation of blood clots. Then after 1 hour, the platelets will continue to synthesize the release of additional growth factors within seven days after that.\textsuperscript{28} The growth factor secreted by PRP binds to the cell membrane’s outer surface in the flaps and wound via transmembrane receptors. Fibroblasts in the periodontal connective tissue have receptors on the cell membrane for growth factor in PRP, which in turn induces endogenous activation of internal signaling proteins that express collagen synthesis.\textsuperscript{29}

TGF-β is very important for soft tissue regeneration and plays a role in the wound healing process (inflammation, proliferation, and maturation). In the inflammatory phase, TGF-β plays a role in recruiting immune cells such as neutrophils and macrophages. In the proliferation phase, TGF-β promotes angiogenesis by stimulating endothelial cell migration, stimulating fibroblast proliferation, encouraging differentiation of transfibroblasts into myofibroblasts. Myofibroblasts are actively involved in the secretion and remodeling of the wound matrix in periodontal tissue cells. Myofibroblasts can originate from connective tissue fibroblasts surrounding wounds in periodontal tissue, mesenchymal cells, and circulating fibrocytes originating from bone marrow and epithelial cells.\textsuperscript{30}

The PDGF can proliferate (mitogenic) and stimulate direct migration of fibroblast (chemotactic) cells which play a role in connective tissue formation by stimulating the formation of fibroblasts in the periodontal ligament by inducing collagen.\textsuperscript{30} Fibronectin is promoted by PDGF with a cell adhesion molecule for proliferation and during healing and inhibits collagen degradation. PDGF synergizes with TGF-β in tissue regeneration by stimulating the proliferation of periodontal ligament fibroblasts.\textsuperscript{31} The freeze-dried PRP method in the study of Murdiasutti et al.\textsuperscript{32} increased PDGF levels, which play a

**Conclusion**

Based on this research, it can be concluded that the application of FD-hPRP increasing the osteoblast number and collagen density in periodontal-tiss treatment, as seen from the results of the histomorphometric analysis in oryctolagus cuniculus rabbits. Further research to examine the effect of FD-hPRP application in bone regeneration as a bone graft material at several observation time points with a longer observation time is recommended to be carried out in the future.

**Acknowledgment**

None.

**Conflict of Interest**

The authors report no conflict of interest.
Increased PDGF levels, which play a role in periodontal ligament fibroblasts, stimulate tissue regeneration by stimulating proliferation and remodeling of the wound matrix. Myofibroblasts can promote healing by releasing TGF-β, which promotes angiogenesis by stimulating cell proliferation, encouraging differentiation during the inflammation phase. TGF-β is very important for soft tissue synthesis, which in turn induces endogenous activation of periodontal connective tissue fibroblasts that have receptors on transmembrane receptors. Fibroblasts in the clot will release growth factors, including fibroblast growth factor (bFGF), including platelet-derived growth factor-AB, which in turn induces endogenous activation of periodontal connective tissue fibroblasts. Oral disease pattern in periodontitis patients with diabetes mellitus using bitewing radiography. J Dentomaxillofac Sci 2018;3: 88-90.

Clinical and biochemical analysis of ligature-induced periodontitis in rats, which can increase TGF-β levels. It is in line with osteoblast migration in vitro compared to the study of Olivia et al. where FD-hPRP can induce osteoblast migration effect of the freeze-dried homologous platelet-rich plasma studied osteogenic potential of freeze-dried homologous platelet-rich plasma on human gingival fibroblast function. Effect of autologous and allogenic PRP on fibroblasts, Oral Dis 2012;18: 494-500.

Reference:
27. Creeper F, Ivanovski S. Effect of autologous and allogenic platelet-rich plasma on human gingival fibroblast

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