ORIGINAL ARTICLE

Freeze-dried Human Platelet-rich Plasma Application Effect in Increase Alveolar Bone Height and Density of Periodontitis Rabbits

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ABSTRACT

Introduction: Regenerative treatment with open flap debridement (OFD) and bone graft material containing growth factors is developed to repair alveolar bone defects due to periodontitis. Freeze-dried human platelet-rich plasma (FD-hPRP) is platelet-rich plasma which is freeze-dried to prolong storage time, simplify application, and increase growth factors number that promote osteoblast proliferation to accelerate bone regeneration. This study examined the effect of FD-hPRP application as a bone graft on the alveolar bone height and density radiographically in periodontitis treatment of Oryctolagus cuniculus rabbits. **Methods:** Twelve-male rabbits were periodontitis induced by ligation technique and Porphyromonas gingivalis lipopolysaccharide injection randomly divided into three groups: 1) OFD, 2) OFD+demineralized freeze-dried bone allograft (DFDBA), 3) OFD+FD-hPRP. OFD was performed in all groups with DFDBA applied in group 2 and FD-hPRP applied in group 3, then subjected to radiographic examination. The alveolar bone height data from Vet-Exam Plus and bone density data from ImageJ then analyzed with a two-way analysis of variance followed by Post Hoc Least Significant Difference test. **Results:** Significant difference ($p<0.05$) between the group 1 and group 2 and 3, and an insignificant difference ($p>0.05$) between the group 2 and 3. **Conclusion:** FD-hPRP application increased alveolar bone height and density radiographically in periodontitis rabbits.

Keywords: Human platelet-rich plasma; Freeze-dried; DFDBA; Open flap debridement; Periodontal regenerative treatment

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INTRODUCTION

Regenerative periodontal treatment to reduce inflammation and restore the function of the periodontal tissue lost due to periodontitis has been carried out through various modalities to achieve optimal oral health. Open flap debridement (OFD) added with bone graft material is the interesting treatments to solve alveolar bone defects. This method fulfills three critical elements of bone formation (osteogenesis, osteoconduction, and osteoinduction) and is also strengthened by the presence of biological mediators, such as growth factors [1, 2, 3, 4].

Demineralized freeze-dried bone allograft is a material containing bone morphogenetic protein (BMP), which can facilitate new bone formation. However, the DFDBA osteoinductive activity is highly dependent on donor characteristics, which makes it difficult to determine the quality standards of DFDBA from one another [5, 6, 7, 8, 9]. Human platelet-rich plasma (hPRP) is a product from healthy donor that play the role in stimulating bone growth [10]. The powder form freeze-dried hPRP (FD-hPRP) is more sterile, simple, and durable, and the freeze-drying process is also known to increase the amount of transforming growth factor-β1 (TGF-β1), which is an essential stimulator in the formation, proliferation, and differentiation of osteoblasts at the bone remodeling stage [11, 12, 13]. Radiographic analysis by measuring the alveolar bone height and density was performed at 4 and 8 weeks after surgery to evaluate the success of mandibular bone regeneration in rabbits [14]. This study aims to determine the effect of FD-hPRP application in periodontitis treatment radiographically of alveolar bone height and

density in *Oryctolagus cuniculus* rabbits.

MATERIALS AND METHODS

This research is a type of quasi-experimental research with a randomized pretest-posttest control only design.

Periodontitis induction procedure

Induction of periodontitis was carried out by ligation technique combined with lipopolysaccharide (LPS) injection of Porphyromonas gingivalis (PG) bacteria. Rabbits were anesthetized using Ketamine HCL 40 mg/kg BW and Xylazine 5 mg/kg BW intramuscularly. Rabbit's cervical mandibular incisors were ligated with 3.0 silk thread. Injection of 0.01 ml LPS in the intragingival interdental mandibular incisors was performed three times a week for six weeks (Figure 1) [15].

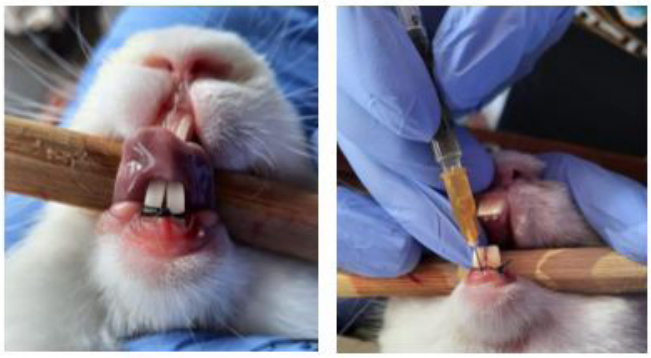

Figure 1 : (a) Rabbit lower incisor ligation with 3.0 silk thread**; (b)** *Porphyromonas gingivalis* lipopolysaccharide injection.

Experimental animal treatment

Twelve rabbits were adapted for one week. Before any intervention, all rabbits were subjected to periapical radiography. Induction of periodontitis was performed on the interdental alveolar bone of the mandibular incisors of twelve rabbits for six weeks, and then a periapical radiograph was performed. Rabbits were randomly divided into three groups, with four rabbits in each group [16]. On all groups, OFD was performed. The OFD surgical procedure was initiated with the anesthetic Ketamine HCl 40 mg/kg BW and Xylazine 5 mg/kg BW intramuscularly. A sulcular incision was made on the labial surface of the mandibular incisor with a surgical blade No. 15. Full-thickness flap reflected with raspatory.

Debridement was performed on soft tissue and alveolar bone using a Gracey curette, then irrigated with saline and distilled water until clean. After OFD, no bone graft material was applied in group 1, then in group 2, DFDBA was applied, and in group 3, FD-hPRP was applied (Figure 2). The bone graft material is condensed into the defects area where the OFD has been performed to cover the alveolar bone properly. The flaps were repositioned using a 4.0 nylon suture with a simple interrupted suturing technique. For 24 hours post-treatment, rabbits were given soft food. Enrofloxacin antibiotics at a dose of 0.1 mg/kg BW and analgesic Meloxicam at a dose of 0.2 mg/kg BW were administered once intramuscular after treatment [17]. Suture removal is performed one week after the surgical procedure.

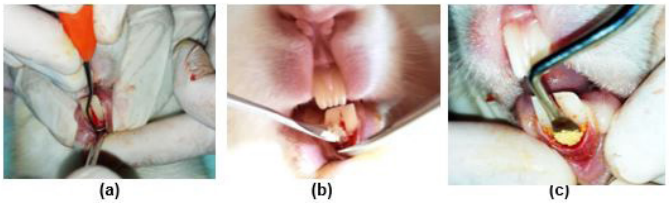

Figure 2 : Open flap debridement **(a)** without the a ddition of bone graft material; **(b)** with the addition of DFDBA; **(c)** with the addition of FD-hPRP.

Alveolar bone height measurement

All rabbits were subjected to periapical radiographic examination in the conditions before the intervention, after the induction of periodontitis, and at the 4 and 8 weeks post-treatment, then the difference was calculated. Periapical radiographic images were scanned using the CR7 Vet Image Plate automatic film processor.

Alveolar bone height was measured through a periapical radiograph by measuringthe distance between cementoenamel junction (CEJ) and alveolar bone crest from the vertical direction using the Vet Exam Plus 7.0.0 Veterinary X-Ray software [18].

Alveolar bone density measurement

Alveolar bone density was measured using periapical radiographs in the region of interest (ROI) in conditions before intervention (ROI 1), after periodontitis induction (ROI 2), week 4 post-treatment (ROI 3), and week 8 post-treatment (ROI 4).

Measurements were carried out using ImageJ software. The percentage of alveolar bone density is calculated by the formula [19]:

Alveolar bone density at four weeks post-treatment = (ROI 3)/(ROI 2) x 100%

Alveolar bone density at week eight post-treatment = (ROI 4)/(ROI 2) x 100%

Ethical Clearance

This study was approved by Research Ethics Committee of the Faculty of Veterinary Medicine, Gadjah Mada University has approved with Certificate of Ethical Eligibility Number 00055/EC-FKH/Eks./2021.

RESULTS

Alveolar bone height

Alveolar bone height results were obtained by measuring the distance between CEJ and alveolar bone crest on the mesial side of the two mandibular incisors through periapical radiograph analysis.

The result of calculating the difference in alveolar bone height is a measure of the increase in alveolar bone height in each treatment type and observation time with the mean and standard deviation as shown in Figure 3.

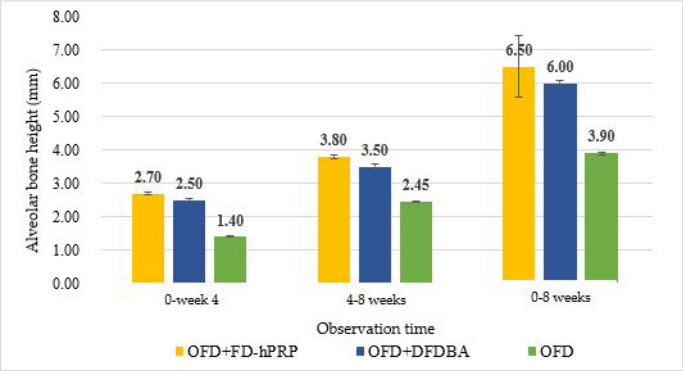

Figure 3 : Mean value of the alveolar bone height increase in each treatment type and observation time.

The descriptive data in Figure 3 shows that the longer observation time, the more significant increase in alveolar bone height was obtained. Mean value of the most significant increase in bone height was seen in the OFD+FD-hPRP group at week 8. Based on the normality and homogeneity test of the data, it can be concluded that the data of the difference in bone height on each treatment type and observation time was normally distributed and homogeneous, so that the data analysis was continued with a two-ways Analysis of Variance (ANOVA).

The two-way ANOVA test results explained that each treatment type, observation time, and the interaction between the treatment type and observation time affected increasing the height of the alveolar bone. Data analysis was then continued with Post Hoc Least Significant Difference (LSD) test. Post Hoc LSD test results showed significant differences in almost all types of treatment and observation time with p-value <0.05, except between OFD+DFDBA and OFD+FD-hPRP groups at each observation time, OFD+DFDBA at week 4-8 and OFD+FD-hPRP at week 0-4, OFD at week 4-8 and OFD+FD-hPRP at week-0-4, OFD at week 4-8 and OFD+DFDBA at week-0-4, OFD at week-0-8 and OFD+FD-hPRP at week 4-8, and OFD at week-0-8 and OFD+DFDBA at week 4-8, with p>0.05.

Alveolar Bone Density

In this research, image processing is done computerized using filters of ImageJ software. Alveolar bone density for each treatment type was compared between week 0 (baseline) with week 4 and week 8 after treatment (Figure 4).

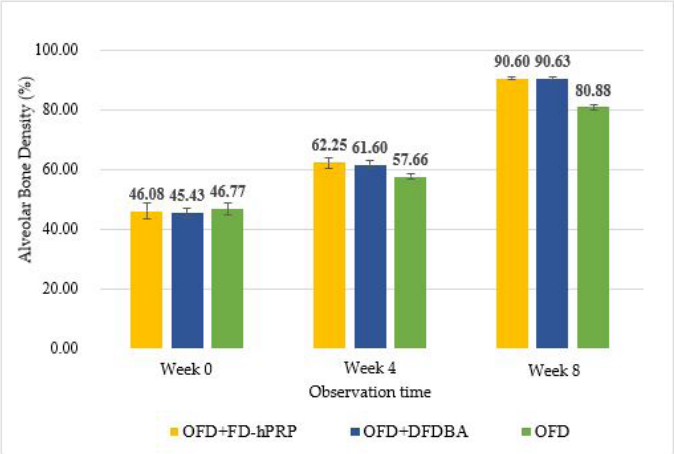

Figure 4 : Mean value of the alveolar bone density in each treatment type and observation time.

Descriptive data on alveolar bone density showed that the longer observation time, the higher alveolar bone density achieved. The highest average was achieved at eight weeks of observation, with the highest bone density value in the OFD+DFDBA group, 90.63. Based on the data normality and homogeneity tests, it can be concluded that the bone density data obtained were normally distributed and homogeneous, so the next statistical test was two-way ANOVA.

The two-way ANOVA statistical test results explained that each treatment type, observation time, and the interaction between the treatment type and the observation time affected increasing alveolar bone density. Data analysis was then continued with the Post Hoc LSD test. Post Hoc LSD test results showed significant differences in almost every treatment type and observation time with p<0.05, except for each treatment type at week 0, OFD+DFDBA and OFD+FD-hPRP at week 4, and OFD+DFDBA and OFD+FD-hPRP at week 8 with p>0.05.

DISCUSSION

Alveolar Bone Height

The week 0 analysis showed a reduction in the alveolar bone height. This is in line with Lin et al. They proved that the PG bacteria LPS injection combined with the ligation technique contributed to alveolar bone destruction and triggered periodontitis quickly [20, 21]. Lipopolysaccharides secreted by pathogenic bacteria induce an immune response in

macrophages and increase the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, thereby triggering osteoclastogenesis that ends in alveolar bone defect [22, 23].

Post Hoc LSD analysis showed no significant difference in the increase in alveolar bone height between OFD+DFDBA and OFD+FD-hPRP groups, both at 0-week 4, 4-8 weeks, and 0-8 weeks of observation. Significant differences were seen when the two groups were compared with the OFD group at the same observation time. This proves that the application of bone graft material, both DFDBA and FD-hPRP post OFD, affects increasing alveolar bone height in the treatment of periodontitis.

The radiographic increase in the height of the alveolar bone is one indicator of the achievement of regeneration [24, 25]. Demineralized freeze-dried bone allograft material has osteoinduction potential related to the amount of BMP-2, -4, and -7, which stimulates mesenchymal cells' differentiation into osteoblasts, thereby affecting new bone regeneration [26, 27]. In comparison, hPRP has many growth factors that modulate inflammatory reactions in the healing process and induce cellular remodeling processes in tissue regeneration. Growth factors in PRP, which include platelet-derived growth factor (PDGF) and TGF-β can promote the regeneration process by increasing cellular chemotaxis and mitosis and promoting the proliferation of regenerative cells. The potential of hPRP in regenerating hard tissue, such as alveolar bone, is also believed to come from the content of other growth factors, such as endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) [28, 29, 30].

Significant differences were also seen between OFD+DFDBA and OFD+FD-hPRP groups when compared between 0-week 4, 4-8 weeks, and 0-8 weeks. This proves that the application of DFDBA and FD-hPRP as post OFD bone graft material increases the alveolar bone height over time. Bone healing is a complex process involving many cell types. In the bone healing phase, various cells' behavior is regulated by various cytokines, growth factors, and unique receptor complexes with varying properties. Physiological interactions during the stages of bone healing will restore bone architecture and function within 6-8 weeks [31].

In Post Hoc LSD analysis, alveolar bone height also showed a non-significant difference between the OFD+DFDBA group 4-8 weeks and OFD+FD-hPRP 0-week 4. This indicates that the difference of alveolar bone height in the treatment type and the observation time has the same pattern. The TGF big family effect on bone healing are coordinated with pro-inflammatory cytokines, extracellular matrix proteins, and the presence of other growth factors [32].

Cottrell et al., in their study on rat femoral fractures, stated that the expression of BMP-2, as contained in DFDBA, was found to be almost four times higher on day 21 compared to the second day of the bone healing process. Likewise, BMP-4 expression was also found to peak at day 21, along with an increase in osteoclast resorption activity during bone remodeling [33]. In contrast, Cho et al. stated that TGF-β1 expression, as contained in PRP, was found before bone defect occurred. TGF-β1 expression was detected to increase during the inflammatory phase on the first day of the bone healing process, and its expression remained the same high on day 28. In addition to TGF-β1, TGF-β2 and -3 expressions were also found in the bone healing phase in smaller amounts, but both still had the same high expression when compared between days 1-7 and day 1-21. Transforming Growth Factor-β2 and -3 are mainly expressed in the reparative phase of bone [34, 35, 36].

The freeze-dried process in hPRP is carried out by breaking the cell membrane through the thermal lysis method, then subliming the liquid hPRP into FD-hPRP in the form of dry powder. Ice formation on the cell membrane causes cells to become easily ruptured, so that platelets release the growth factors contained in them [37, 38]. Murdiastuti et al. stated in their research results that hPRP that has gone through the freeze-drying process has a higher content of TGF-β1 so that it can stimulate and accelerate the process of alveolar bone regeneration [13].

Alveolar Bone Density

Post Hoc LSD analysis results on alveolar bone density showed no significant difference between the three treatment types at week 0. At that time, bone density was reduced seen in all rabbits radiographically. This proves that all rabbits experienced the same periodontitis condition after being induced by ligation technique and LPS injection of PG bacteria for six weeks.

Significant differences between OFD and OFD+DFDBA groups and between OFD and OFD+FD-hPRP groups, at 4 and 8 weeks of observation also showed in this study. There was also a significant difference when the OFD+DFDBA and OFD+FD-hPRP groups were compared between 4 and 8 weeks of observation. At the observation time, there was no significant difference between OFD+DFDBA and OFD+FD-hPRP groups. The results prove that the application of DFDBA and

FD-hPRP as post OFD bone graft material affects increasing alveolar bone density in the treatment of periodontitis, as evidenced by the thickening of the alveolar bone trabeculae on the periapical radiograph. Alveolar bone healing after periodontitis treatment radiographically appears from the compaction of the bone image, which will increase the absorption of X-ray photons. Newly formed bone will absorb more X-rays so that only a few X-rays will hit the sensitive crystals on the film and show a radiopaque appearance [39].

The key of periodontal regenerative treatment is to stimulate progenitor cells to re-occupy the defects area. Growth factors are vital modulators that induce migration, attachment, proliferation, and differentiation of periodontal tissue progenitor cells [40]. Stimulation of BMP-2, -4, and -7, such as those contained in DFDBA, can affect the major transcription factors involved in osteoblast differentiation during osteogenesis, thereby increasing trabecular thickness. Trabeculae are considered important in radiographic images because the loss of trabeculae indicates reduced alveolar bone density. An increase in the number of trabeculae and a reduction in the trabecular separator due to BMP stimulation began to be seen on day 14 and became more pronounced on day 21. The alveolar bone remodeling phase is characterized by an increase in osteoblast stability, which indicates an increase in bone density [41, 42]. Gawish et al., in their research on animal experimental, proved that DFDBA could produce new bone formation on day 21. The radiographic appearance at six weeks after DFDBA application to bone defect showed an increase in the thickness of the alveolar bone trabeculae along with the loss of osteoclasts in Howship's lacunae [43, 44].

Bone grafting with FD-hPRP is a more straightforward and physiological method of applying growth factors stimulating bone regeneration. The growth factors PDGF and TGF-β contained in PRP are believed to be physiological secretions of platelets, which are synergistically capable of initiating the alveolar bone healing process. Transforming growth factor-β1 is a cell-signaling molecule that plays a role in cell growth, migration, and differentiation in bone regeneration [45, 46]. Several *in vitro* studies suggest that the primary role of PDGF is in osteoblast proliferation. At the same time, TGF-β acts as a cellular differentiation agent as a marker of cell mineralization expression when incubated with pre-osteoblastic cells. This shows that TGF-β can support osteoblast differentiation and produce molecules involved in osteoblast adhesion and angiogenic processes, so PRP is suitable for use in the early stages of bone healing because it will accelerate mineralization and alveolar bone density [47].

Freeze-dried human platelet-rich plasma preparations were prepared through a sterilization procedure to avoid bacterial infection, extend storage time, and maintain material bioactivity so that biological activity and growth factors in FD-hPRP were maintained as fresh PRP [48]. This has been proven by Murdiastuti et al. in their in vitro study evaluating the osteogenic potential of FD-hPRP. The results showed that FD-hPRP could affect the process of osteogenesis, as seen from osteoblast differentiation during seven days of incubation. A differentiation process can be identified by the morphological changes and an increase in the number of osteocytes in the bone cell culture population [49]. This study follows the statement of Nakatani et al., who have proven that bone regeneration achieved after *in vivo* application of FD-hPRP can resemble fresh PRP [50].

CONCLUSION

Based on this research, it can be concluded that the application of FD-hPRP post OFD procedure increasing the alveolar bone height and density in periodontitis treatment, as seen from the results of the radiographic analysis in Oryctolagus cuniculus rabbits. Further research to examine the effect of FD-hPRP application as a bone graft material on alveolar bone regeneration at several observation time points with a longer observation time is recommended to be carried out in the future.

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