ORIGINAL ARTICLE

The Osteopontin Levels between Activated Collagen - Platelet-Rich Plasma and Platelet-Rich Plasma Gel on Flap Surgery

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ABSTRACT

Introduction: Platelet-Rich Plasma (PRP) is an autologous blood (platelet concentrates) that able to modulate wound healing in periodontal diseases. Increasing of growth factor number and effectiveness in PRP need activator. Activator PRP that safe and widely used is collagen and $CaCl_2$ (gel). Osteopontin (OPN) is produced by osteoblasts that can stimulate bone remodeling and be found in gingival crevicular fluid (GCF). The research aim was to determine the difference of OPN level in GCF between PRP- collagen and PRP gel on flap surgical therapy. **Methods:** Samples were taken from the GCF on 20 points of infrabony pockets were divided into 2 groups. Open flap debridement (OFD) was used and combined with PRP-collagen for the first group and for the second group was OFD+PRP gel, which examined on the day, 14, and 21 by measured GCF OPN level used Human OPN Elisa Kit. The collected data were analyzed by Friedman and Wilcoxon test. **Results:** This study indicated that there was increasing of OPN level in both groups on the day, day14, and day 21 but no significant differences between both groups on day14 and day 21. **Conclusion:** There was no difference of OPN level in GCF between PRP-collagen and PRP gel on flap surgical therapy.

Keywords: Osteopontin, Gingival crevicular fluid, Platelet-rich plasma, Collagen

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INTRODUCTION

Chronic periodontitis is an inflammation of the periodontal tissues caused by bacteria in dental plaque. In susceptible individuals, this inflammation process will result in the soft and hard tissue damage so that can develop periodontal pockets and serve the reservoir for bacteria. This condition is often not noticed by the patient and can cause discomfort to the teeth, cosmetic problems (such as tooth migration and gingival recession), the tendency of abscess formation in the pockets, and tooth loss. Bone destruction in periodontitis is often shaped like a crater with damage around the tooth root, called as the infrabony pocket with the base is more apical than the alveolar bone crest, and the walls are located in pockets between the teeth and alveolar bones. Infrabony pocket needs more complex treatment (1,2).

Several periodontal regeneration techniques are used for the treatment of infrabony pockets. Flap surgical therapy is one of the most frequently performed, especially for the medium and deep pockets. Surgical flap for therapy of infrabony pocket have a purpose: 1) to improve accessibility to the root surface for scaling root planing; 2) to eliminate or reduce the pocket depth by pocket wall resection; 3) to achieve access to the surgical bone if needed; and 4) to expose the area to perform the method of periodontal regenerative (2). Treatment with periodontal flap surgery usually results in the repair process and not a regeneration. This was proven by histology examination, with the discovery of long junction epithelium in the alveolar bone with the tooth surface (3). Tissue engineering is a biomedical engineering involved in the manipulation of artificial cells to increase regeneration of tissues and organs, which is an advancement in the medical field and thrive in the 21st century. The aim of tissue engineering is to regenerate the natural tissue of the living cells to replace missing tissues and organs. The main component of tissue engineering is cells, scaffolds, and growth factor (4).

Platelet rich plasma is obtained from patients' blood centrifugation and contains growth factors which is

affect in the healing process that plays in role of tissue repair mechanism (5). In surgery, PRP can provide many benefits, which reduces bleeding and promotes healing of soft tissue and bone regeneration (6), however PRP application in liquid form into the wound in the oral cavity often makes a significant loss amounts of PRP, if not in the form of gelatin through the clotting mechanism. Gelatin can be achieved by using bovine thrombin. Therapy using bovine thrombin have complications in antibody. Bovine thrombin is a strong activator of platelets that can lead to the development of antibodies which can lead to serious clinical problems such as severe postoperative bleeding until a lupus-like autoimmune disease in experiment animals (7). Smith et al. (2007) stated that bovine thrombin become scarce and are not available, so must be find alternative options to change (8).

The bovine thrombin can be replaced by collagen because collagen is also involved in the intrinsic coagulation cascade and is widely used as biomaterial (9). PRP clot which is activated with type I collagen is more stable against retraction compared by thrombin, this description may be important in relation to the PRP clot as a scaffold wound healing. These findings suggest type I collagen is an activator that is safe, easy to obtain, and not expensive (7). Platelet rich plasma can also be activated by $CaCl_2$ and / or thrombin.

The growth factor which is in is a rapid release of this will be lost before stimulating cells. In platelet gel formed using $CaCl_2$ will release gradually growth factor. Calcium chloride activates and form a clot PRP with thrombin formed from prothrombin autogenous which led the formation of fibrin matrix that will release the growth factors over 7 days (10).

Osteopontin (OPN) is a non-collagen protein, high-phosphorylated, sialic acid rich, and has ties RGD (arginine-glycine-aspartic acid). It is found in the kidneys, blood, mammary glands, salivary glands, and calculus (11.12). Osteopontin is used as the early marker of bone formation (13). Osteopontin expressed during the early phase of bone cell differentiation in cultured osteoblasts, with the highest levels after mineralization begins, and this expression remained in high levels in the final phase mineralization (11,14). Kido et al. (2001) were first isolated the molecules in the OPN gingival crevicular fluid. They had founded that levels of OPN ± 1.48 ng/ side on a sulcus depth of 1 mm, 43.42 ng / side on the side of the disease (with probing pocket depth ≥ 4mm) and 1.93 ng / side on the healthy side (PPD ≤ 3 mm) (15).

The previous studies showed the effectiveness of collagen and $CaCl_2$ and/or Thrombin as activators in PRP preparation, however the novelty of this study was on differences in osteopontin levels of gingival fluid between PRP-collagen and PRP gels combination with flap surgical in an infrabony pocket therapy has never been done.

This study was aimed to determine differences in osteopontin levels between the addition of PRP-collagen and PRP gel in the treatment of surgical flap.

MATERIALS AND METHODS

This research had been approved by the Ethics Committee, Faculty Dentistry, Universitas Gadjah Mada, Indonesia with registration number 00274/KKEP/FKG-UGM/EC/2017. The research subjects were patients who came to the Periodontology clinic who fulfill the following inclusion criteria that used as baseline characteristics of the patients: a. Patients aged (30 to 55) years old with Chronic periodontitis, infrabony pocket 5 mm to 7 mm; b. Not patients of cancer, obesity, Diabetes mellitus, coronary artery disease, Smoking or drinking alcohol; consuming long-term drugs; c. Willing to agree and sign an informed consent; to do a surgical flap and willing to participate in this study until complete. After had got several patients that fulfill the inclusion criteria, then randomized to take the sample. The samples consisted of 20 points of infrabony pockets in chronic periodontitis, who were divided into two treatment groups. Group A was 10 pockets infrabony treated with flap surgery and PRP-collagen. Group B was 10 pockets infrabony treated with flap surgery and PRP gel. Samples had taken at the time before surgery (baseline), day 14, and day 21 after surgery. The study was conducted through the following steps: 1). Ethical clearance was obtained by submitting a proposal to the ethics committee dentistry; 2). Preparation of PRP: one hour before surgery, 9 mL the blood of the subject was taken from ante-cubital vein and put into a 10 mL tube that contained of 1 mL 3.8 % sodium citrate anticoagulant. The first centrifugation performed at 2400 rpm for 10 minutes. Blood clots in the tube would be divided into two layers: the bottom layer consisted red blood cell and the top layer contained buffy coat and blood plasma, which was a collection of platelets. The top layer aspirated with a pipette and put into another tube, centrifuged at 3600 rpm 15 minutes. After the top layer aspirated as much as 2/3 of the volume of the supernatant and the bottom third contains platelets, a small number of blood cells and blood plasma, called PRP. 3). Platelet rich plasma gel was prepared immediately before application. In the first stage of making autologous thrombin by taking 4 mL of autologous blood into the tube and allowed to stand for ± 30 minutes until coagulation occurs, then centrifuged at 3200 rpm for 2 minutes. The top of the supernatant was collected as autologous thrombin. Making the PRP gel by mixing 0.1 ml of autologous thrombin with 0.03 mL of 10 % $CaCl_2$. PRP and autologous thrombin ratio is 10:1.

Samples were obtained from the gingival crevicular fluid. Areas that would be taken samples were isolated

using cotton rolls and dried. Samples were taken using paper points (size 30) for

30 seconds and put into a safe-lock which contains 500 mL of phosphate-buffered saline (PBS). Then the samples were centrifuged at 2500 rpm for 20 minutes, the paper points separated, samples were stored at –70 °C until used (samples will be analyzed using Elisa Kit Human Osteopontin Quantikine (RnD)). The gingival crevicular fluid collection prior to surgical therapy flap and recorded as the initial data of the study (baseline). Then the flap surgical performed by adding of PRP- collagen and PRP gel. The surgical procedure of periodontal flap starts after PRP was made. Anesthetic used is a local anesthetic, vertical and sulcular incision of full thickness flap was done and the flap was elevated, and debridement performed on the defect area with SRP and curettage. Tetracycline HCl solution 75 mg / mL was applied to the hard tissue for 3 minutes and rinsed by distilled water then application of PRP that was activated by collagen and PRP gel on the area of operation.

Flap was returned and sutured with vertical mattress and interrupted techniques, then closed with periodontal dressing. Patients are given antibiotics amoxycilin 500 mg for 5 days, the patient was also given an analgesic and anti-inflammatory. Before leaving, the patient is given instructions and an explanation of how to care for their teeth and mouth postoperatively, controls carried out 7 days later to remove the periodontal dressing and control at day 14 for release suturing. Oral hygiene control and wound healing done once a week for 4 weeks after periodontal flap surgery. On day 14 and 21 after the treatment, the gingival crevicular fluid was taken and the results were recorded as the data. The levels of OPN measured by Elisa test and the results were read by ELISA reader. The ELISA procedure using Elisa Kit Human Osteopontin Quantikine (R&D Systems, Minneapolis, Minnesota).

RESULTS

Data were taken by spectrophotometer result at a wavelength of 450 nm in the form of optical density (OD). Optical density was converted into a standard curve to obtain the value of the levels of osteopontin (OPN).

Figure 1 showed the lowest mean of OPN GCF levels was in the group OFD + PRP gel at day (baseline) (0.038 ± 0.331 ng/ ml). The highest means levels of OPN in the gingival crevicular fluid at OFD + PRP- collagen on day 21 (0.394 ± 0.030 ng/ml).

The result of this study as intended to answer the hypothesis, whether there were differences in the levels of OPN GCF between the flap surgical therapy combined with PRP- collagen and PRP gel using two-ways ANOVA. Terms for using parametric tests are

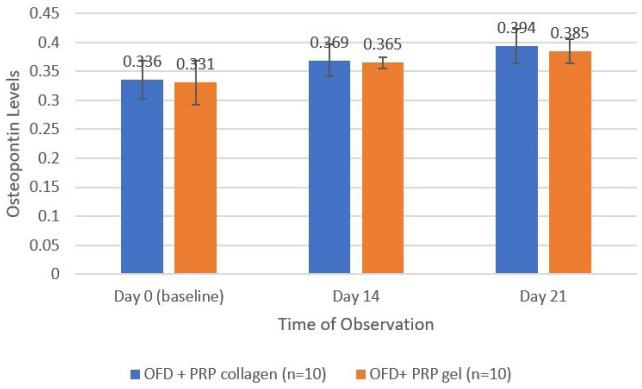

Figure 1: Graft of the gingival crevicular fluid osteopontin levels (ng/ml) according to time and group

normally distributed data and homogeneous. The normality test (Shapiro-Wilk test) showed a significant level of OFD + PRP- collagen group was $p > 0.05$ and $p < 0.05$ in the group of OFD + PRP gel. This means that the data distribution was not normal in the group OFD + PRP gel. Results of homogeneity test of osteopontin GCF levels showed a significance level of $p > 0.05$. This indicates a homogenous research data. In the Shapiro-Wilk test data obtained OFD + gel PRP group were not normally distributed so that the statistical test followed by non-parametric Friedman test.

The significant level of Friedman test was $p = 0.000$ ($p < 0.05$) indicated that there were significant differences in the levels of OPN GCF between the treatment groups and also between the time of observation (Table I). To know inter the groups and the time of observation had significant differences, could statistically use the Post Hoc followed by Wilcoxon tests.

Table I: Results of Friedman test between group and time

	F	Significance
Friedman test	5	0,000*

*p< 0,05 significance

Table II shows the significant differences in the levels of OPN between groups OFD + PRP collagen on the day and day 14, the day and day 21, day 14 and day 21. There were also significant differences in levels of OPN that the group OFD + PRP gel at the day and

Table II: Results of the Wilcoxon test between OFD + PRP-collagen and OFD + PRP gel on the day (baseline), day 14 and day 21

	12	13	21	22	23
11	0,005*	0,005*	1,000	0,008*	0,008*
12		0,005*	0,005*	0,593	0,168
13			0,005*	0,011*	0,441
21				0,008*	0,005*
22					0,005*

*p< 0,05 significance
Notes:
11: OFD+PRP collagen day 0 (baseline)
12: OFD+PRP collagen day 14
13: OFD+PRP collagen day 21
21: OFD+PRP gel day 0
22: OFD+ PRP gel day 14
23: OFD+ PRP gel day 21

day 14, day 14 and day 21, the day and day 21. There were differences in the levels of OPN significantly to the group OFD + PRP gel day 0 with OFD + PRP-collagen day 14 and 21, OFD + PRP gel day 14 and OFD + PRP-collagen day 0 and 21, and OFD + PRP gel day 21 and OFD + PRP-collagen day 0. In Table II showed there was no significant difference of OPN levels between OFD + PRP-collagen and OFD+PRP gel groups at day 14 and at day 21.

DISCUSSION

Figure 1 showed the pattern of elevated levels OPN GCF based the observation time of the OFD + PRP-collagen group as well as the OFD + PRP gel group. This was consistent with the statement of Denhardt et al. (2001) and Kullmer et al. (2006) that OPN in osteoblast cell cultures will be expressed during the early phase of bone cell differentiation, with the highest level after the mineralization begin and this expression remains in the high level during the final phase of mineralization (day 14- 21) (11,16). Rodriguez et al. (2014) suggest that OPN is strongly associated with the mineral phase of reinforcement and forming osteoblasts. Osteopontin expressed at or near the time of mineralization to increase the mineralized collagen matrix to intrafibrillar (10).

Jankovska et al. (2009) and Neve et al. (2010) states that OPN is phosphorylation acid glycoprotein secreted by osteoblasts and found in large numbers in the immature bone on bone edges. Osteopontin is initial marker and osteocalcin as final marker at bone matrix formation (11,17). Osteocalcin (OC) showed new bone matrix formation on day 45 and day 60 which will depict adult bone (osteocytes) (18). Source OPN GCF come from the neighboring tissue, such as alveolar bone and cementum, macrophages, blood, and salivary glands may explain the OPN GCF in periodontal tissue without inflammation (19). Increased levels of OPN in the gingival crevicular fluid can also be followed by an increase in blood plasma OPN levels caused by excessive spending OPN on periodontal tissue or production by circulating activated by macrophages (20).

In table I, the Friedman test obtained $p = 0,000$ ($p < 0.05$) indicates there were significant differences the OPN GCF levels between the treatment groups (OFD + PRP-collagen and OFD + PRP gel) and between groups of observation time. The Wilcoxon test results showed that there were significant increased OPN GCF levels for OFD + PRP-collagen and OFD + PRP gel groups at the day, day 14 to day 21. This could be due to an activator PRP, collagen and $CaCl_2$ (gel) can help releasing growth factors in PRP regularly and for longer. Fufa et al. (2008) reported that type I collagen effectively stimulate the PDGF- AB and VEGF release between day 1 to day 10 and can stabilize the clot PRP against excessive depreciation (7). Clot activates collagen will shrink by about 50 % in 24 hours and then stabilized until more than 10 days. Collagen can also be applied as scaffold that serves as osteoconductive effect in bone regeneration (10). The PRP - activated thrombin could induce releasing growth factors within a few hours after activation. However, PRP-activated collagen will gradually release growth factors within several days. The time difference of growth factors releasing base on platelet activation mechanism. Platelets are activated by collagen and collagen must follow will then be activated by other receptors. This process requires longer mechanisms for platelet activation compared enzymes in the division process of platelet activation by thrombin (7,9).

The PRP gel is made by mixing PRP with autologous thrombin and calcium. The adding thrombin and calcium of PRP will automatically activate the granules alpha to release growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) 1 and 2, and vascular endothelial growth factor (VEGF) that play a role of angiogenesis in wound healing process. The VEGF can stimulate endothelial cell proliferation. The PDGF will stimulate revascularization, the synthesis of collagen, bone formation and tissue healing (21,22).

According Mazzucco et al. (2009), PRP gel will result in mixed networks with most of PDGF after 24 hours to 7 days. In PRP gel, a lot of IGF-I to be issued at the end of the growth factor release phase. The PRP gel will release growth factor more than 7 days (23). Use of $CaCl_2$ resulted in shrinkage of blood clots PRP smaller so favorable to the growth factor to be released in a longer time (24). Gel platelets are formed using $CaCl_2$ releases growth factors slowly. Calcium chloride will activate and form a clot PRP by making autogenous thrombin from pro thrombin and create a matrix of loose fibrin, then release growth factors over 7 days (10).

In table II for comparison OPN levels of gingival crevicular fluid between OFD + PRP collagen and OFD + PRP gel did not reveal any differences in the levels of osteopontin significant gingival sulcus fluid on day 14 and day 21. Osteopontin are at an early stage during the development of bones and plays a part in increasing osteoblast adhesion to the extracellular matrix of (18). The growth factors play in the osteoblast adhesion to the extracellular matrix is PDGF and TGF-β1 (10). Activation of PRP using collagen produces gradual release is longer than the other PRP activator. Harrison et al. (2011) reported that activators just change the time of release but does not change the final concentration in the release PDGF (9). Research conducted Fufa et al. (2008) also reported a gradual release of TGF-β1 with an activator of thrombin or collagen have the same amount significant (7). Total concentration of the final release of PDGF and TGF-β1 same can cause no significant difference OPN levels of gingival sulcus fluid between collagen-PRP group and PRP gel when compared on day 14 and day 21. This results also supported by our research that

the study used clinical parameters by Pocket Depth (PD), Attachment Level (CAL) and alveolar bone height (unpublish data). It could be concluded that the results of infrabony pocket treatment with PRP-collagen were similar with PRP gel in decreased pocket.

CONCLUSION

There were no differences in osteopontin gingival crevicular fluid levels between the addition of platelet-rich plasma collagen and platelet-rich plasma gel in the treatment of surgical flap.

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