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Effectiveness freeze dried platelet rich plasma combination of hyaluronic acid after periodontal surgical healing: an experimental study on animal model

Eficácia da combinação de plasma rico em plaquetas liofilizado e ácido hialurônico após cicatrização cirúrgica periodontal: um estudo experimental em modelo animal

Pitu WULANDARI[1], Denny SATRIA[2], Martina AMALIA[1], BUDI[3], Rio SIMANJUNTAK[3]

1 - Universitas Sumatera Utara, Faculty of Dentistry, Department of Periodontics, Medan, Indonesia
2 - Universitas Sumatera Utara, Department of Pharmaceutical Biology, Medan, Indonesia
3 - Universitas Sumatera Utara, Faculty of Dentistry, Periodontics Residency Program, Medan, Indonesia

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ABSTRACT

Objective: the study aimed to investigate the effectiveness of the combined application freeze-dried platelet-rich plasma (FD-PRP) and hyaluronic acid (HA) in healing after periodontal surgery. **Material and Methods:** This is an experimental laboratory research with a design pre and post-test only control group design. Freeze-dried platelet-rich plasma and HA were applied at periodontal surgical sites in periodontitis induced Wistar rats' mandibulary incisors. Bleeding on probing (BOP) was observed as a clinical aspect. *Porphyromonas gingivalis* was examined and counted. Meanwhile, the healing process was seen by examining fibroblasts and Platelet-Derived Growth Factor-BB (PDGF-BB) in histology and immunohistochemistry. In this study, BOP decreased faster after applying FD-PRP and HA. Data were processed by ANOVA and Kruskal-Wallis test. **Results:** Clinical examination by measuring BOP level showed better inflammation resolution at 14th day (p=0.007). *Porphyromonas gingivalis (P. gingivalis)* count was also reduced in the treatment group (p=0.001 at 3rd and 14th day). The histology aspect by measuring fibroblast count (p=0.025 at 3rd day and p=0.001 at 14th day) and immunohistochemistry by measuring PDGF-BB expression (p=0.014 at 3rd day and p=0.030 at 14th day) showed better tissue healing. **Conclusion:** combination of FD-PRP and HA has accelerated the healing of periodontal surgical wounds.

KEYWORDS

Fibroblast; Hyaluronic acid; Periodontitis; Platelet-rich plasma; *Porphyromonas gingivalis*.

RESUMO

Objetivo: o estudo teve como objetivo investigar a eficácia da aplicação combinada de plasma rico em plaquetas liofilizado (FD-PRP) e ácido hialurônico (AH) na cicatrização após cirurgia periodontal. **Material e Métodos:** Trata-se de uma pesquisa experimental com um delineamento pré e pós-teste apenas de grupo controle. Plasma rico em plaquetas liofilizado e AH foram aplicados em sítios cirúrgicos periodontais induzidos por periodontite em incisivos mandibulares de ratos Wistar. Sangramento à sondagem (SS) foi observado como aspecto clínico. *Porphyromonas gingivalis* foi examinado e contado. Enquanto isso, o processo de cicatrização foi observado por exame de fibroblastos e fator de crescimento derivado de plaquetas-BB (PDGF-BB) em histologia e imuno-histoquímica. Neste estudo, o SS diminuiu mais rapidamente após a aplicação de FD-PRP e AH. Os dados foram processados por ANOVA e teste de Kruskal-Wallis. **Resultados:** O exame clínico do nível de SS mostrou melhor resolução da inflamação no 14º dia (p=0,007). A contagem de *Porphyromonas gingivalis* (*P. gingivalis*) também foi reduzida no grupo de tratamento (p=0,001 no 3º e 14º dias).

O aspecto histológico pela contagem de fibroblastos (p=0,025 no 3º dia e p=0,001 no 14º dia) e imunohistoquímico pela contagem da expressão de PDGF-BB (p=0,014 no 3º dia e p=0,030 no 14º dia) mostraram melhor cicatrização tecidual. **Conclusão:** a combinação de FD-PRP e AH acelerou a cicatrização de feridas cirúrgicas periodontais.

PALAVRAS-CHAVE

Fibroblasto; Ácido hialurônico; Periodontite; Plasma rico em plaquetas; *Porphyromonas gingivalis*.

INTRODUCTION

One of the critical aspects of the wound healing response in the periodontium is the regeneration of the cementum-connected collagen fibers that cover the tooth roots, gingival connective tissue and alveolar bone. Immediately following injury to the periodontal tissues, a vascular cascade is activated to control local arteriolar and venous bleeding. The platelet plug is formed by a mixture of fibrin and fibronectin which actively plays a mechanical role in supporting and as a necessary framework for tissue healing. Through the granules, locally activated platelets will release growth factors, cytokines and chemokines that will stimulate cell proliferation, adhesion and migration. Platelet rich plasma (PRP) is an autologous platelet containing small volumes of plasma, which is enriched with growth factors and proteins that stimulate cellular processes such as chemotaxis, mitogenesis, cell differentiation and angiogenesis [1,2].

Platelets rich plasma have shown beneficial effects by relieving postoperative discomfort and preventing infection, as well as acting as anti-inflammatory and anti-bacterial. In recent years, interest in the use of PRP in the treatment of tissue infections has increased [3]. Although much progress has been made in clinical practice, the treatment of chronic wounds and bone infections remains a challenge. Conventional treatments for treating bone infections include debridement of the infectious wound, use of antibiotics, and restoration of the soft tissue covering the wound infecting the bone. Often these treatments are ineffective in producing a timely resolution of infection, which can cause significant morbidity and an economic burden for the patient [4].

Bacterial attachment and growth is an important initial step in colonization. Platelet rich plasma can also inhibit the attachment and growth of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* which are periodontal pathogens but platelet rich fibrin (PRF) cannot kill these microorganisms. Platelet rich plasma can inhibit the growth of microorganisms in vitro such as *Enterococcus faecalis, Candida albicans, Streptococcus agalactiae* and *Streptococcus oralis*. Importantly, PRP only exerts antibacterial properties during activation and is effective against a wide range of gram-positive and gram-negative bacteria [5,6]. Platelet rich plasma also contains leukocytes which play a significant role as antibacterial. The antibacterial effect of PRP is seen after activation, however, PRP appears weak and short-lived when used alone. Platelet rich plasma acts synergistically with antibiotics and can be considered as an adjunct treatment for pathogenic infections especially in antibiotic-resistant cases [6].

The role of HA in periodontal disease is through its anti-inflammatory and anti-bacterial effects. Tissue healing can be exploited using collaboration in periodontitis therapy. Hyaluronic acid is an extracellular part of connective tissue that functions as a barrier from bacterial plaque and fulfills various extracellular functions that are important in maintaining healthy gingival tissue. It also has a multifunctional role in the wound healing process and its mechanism is similar to that of wound healing in periodontal tissues [7]. Hyaluronic acid is further involved in the activation of inflammatory cells such as polymorphonuclear leukocytes (PMNs), macrophage functions, including their migration to the wound site, phagocytosis and killing invading pathogenic microbes to prevent colonization and proliferation anaerobic pathogens in the gingival crevice and adjacent periodontal tissues [8]. Srisuwan et al. [9] demonstrated that HA has potential in directed tissue development, and this material is engineered for various medical interests.

The study aimed to investigate the effectiveness of the combined application freeze-dried platelet-rich plasma (FD-PRP) and hyaluronic acid (HA) in healing after periodontal surgery. A combination of the use of PRP and HA in the biofunctional scaffold can provide many advantages in wound healing through the speed of healing compared to advanced dressings, and the use of PRP alone as a biostimulator or HA as a scaffold and this will also reduce postoperative pain. Combination of the two is expected to be faster in closing the wound with the best esthetics [10].

MATERIAL AND METHODS

Experimental animals

This study tested the effectiveness of combination FD-PRP and HA after periodontal surgery healing in male Wistar rats, aged 8-12 weeks, weighing 200-250 grams and having a healthy body condition, induced by lipopolysaccharide bacteria *P. gingivalis* strain ATCC 33277. Calculation of the number of samples using the Federer formula with a total sample of 34 rats. This research has been approved by the Research Ethics Commission of the Faculty of Mathematics and Natural Sciences in Universitas Sumatera Utara (Animal Research Ethics Committees/AREC) with letter No. 0350/KEPH-FMIPA/2021. Experimental animals were adapted and kept in cages placed in a room with sufficient airflow and light for 10 days. During the adaptation period, the animals were monitored for food and drink intake, urination, weight gain or loss and general behavior of the animals to evaluate the stress level of the animals. Animal cages are cleaned every day of dirt and food residue. Animals were fed food and water three times a day during the trial period.

Periodontitis induction

Before doing periodontal treatment, induction of periodontal pathogens was carried out in experimental animals so that the experimental animals suffer from periodontitis.The procedure begins with administering anesthesia via intramuscular injection with a solution of 10% Ketamine and 2% Xylazine (2: 1), 0.12 ml/100 g body weight. The ligature was inserted into the gingival sulcus of the lower incisors in an "8" shape using a sterile non-resorbable silk thread combined with a single wire, and the animals were given a high carbohydrate diet. One week later, the induction of *P. gingivalis* bacteria ATCC 33277 was carried out in the gingival sulcus area. *Porphyromonas gingivalis* is a periodontal pathogenic bacteria that can cause periodontal destruction. After ten days, swabs were taken for microbiological culture examination to ensure the mice had been infected with *P. gingivalis* bacteria. The high-carbohydrate diet was stopped after the mice were confirmed to have been infected with *P. gingivalis* bacteria, and the ligatures were released and ready to be treated.

Periodontal treatment

After the experimental animal suffers from periodontitis, it is followed by the surgical stage. The samples were divided into two negative control groups, two positive control groups, and six treatment groups where all groups had been induced with *P. gingivalis*. The negative control was not given treatment; the positive control was given flap surgery only using the modified Widman flap method and the other 3 groups were given flap surgery with single and combined drug applications. This procedure was carried out at the Animal House, Faculty of Mathematics and Natural Sciences in Universitas Sumatera Utara. The internal bevel scallop incision to the cristae alveolar; the flap is elevated. The flap can be thinned to allow adaptation to the gingival density around the teeth, and a sulcular incision is made in the gingival sulcus to release connective tissue from the root surface. The gingival margin and granulation tissue were removed with a curette; then, root planing was carried out before the material was added.

Hyaluronic acid used is HA 0.2% obtained from preparations with the gengigel brand, while the FD-PRP used is a product of the Biotechnology Laboratory, Faculty of Dentistry in Universitas Gadjah Mada. The manufacture of a combination of FD-PRP and HA was carried out at the Integrated Laboratory, Faculty of Pharmacy in Universitas Sumatera Utara using a chitosan-based gel. All tools that will be used for the manufacture of chitosan hydrogel based gels were sterilized in an oven at 170°C for 1 hour. Aseptic preparation of the gel was carried out in a laminar air flow cabinet, 2 gram of chitosan was put into a stamper then added with a 1% lactic acid solution that had been diluted as much as 50 ml and stirred slowly to form a 4% chitosan hydrogel using a magnetic stirrer [11].

Clinical, microbiological, histological and immunohistochemical examinations

The clinical parameters examined were BOP on days 3 and 14 using a periodontal probe inserted into the gingival sulcus of the lower incisor. In addition, a swab was also performed on the gingival sulcus to assess the levels of *P. gingivalis* by counting the number of bacterial colonies on days three and 14. Then the rats were euthanized and the periodontal tissues were taken and immersed in 10% formalin buffer for 2-4 hours. Furthermore, the specimen was processed at the Histology Laboratory, Faculty of Medicine

in Universitas Sumatera Utara to obtain preparations with hematoxylin-eosin staining. After that, the expression of is also observed PDGF-BB by immunohistochemical staining. Immunohistochemical quantitative analysis was performed under a binocular microscope.

Statistical analysis

The data obtained from the study are quantitative data in the form of clinical, microbiological, histological and immunohistochemical parameters before and after treatment on days 3 and 14. The data were processed by ANOVA and Kruskal-Wallis test to see the difference between the groups on the third and fourteenth day.

RESULTS

Based on the results of microbiological examination, the average score of the lowest number of bacteria was found in the positive control group on days 3 and 14. Statistically it was shown that the treatment significantly reduced the number of bacteria scores in all groups on days 3 and 14 (Table I).

Based on histological examination, the highest mean fibroblast count score was found in the AH and FD-PRP combination group on day 3 and the lowest on day 14. This indicates that the treatment significantly increased the fibroblast count score on day 3 and 14 statistically (Table II).

Based on immunohistochemical examination, the highest average PDGF-BB expression score was found in the FD-PRP group and the AH and FD-PRP combination on day 3. Statistically, there was a significant difference in mean PDGF-BB expression in all groups. The treatment caused PDGF-BB expression to increase significantly on the 3rd day. However, on day 14, the highest PDGF-BB expression score was seen in the negative control group. Statistically, the treatment also increased PDGF-BB expression on days 3 and 14 (Table III, Figure 1 and 2).

Based on clinical examination, the highest average BOP score was found in the positive control group on days 3 and 14. The lowest BOP score was found in the FD-PRP group on day 3 and in all treatment groups on day 14. Results of statistical analysis on day 3 showed that the treatment did not significantly reduce the BOP score, but this was different on day 14 which showed that the treatment significantly reduced the BOP score (Table IV).

Table I - Differences of the number of bacterial colonies on days 3 and 14.

Group	Number of bacterial colonies			
	Day 3		Day 14	
	$\bar{x}$ ± SD	p value	$\bar{x}$ ± SD	p value
Control (-)	> 300.00 ± 0.000		> 300.00 ± 0.000	
Control (+)	149.667 ± 25.697		59.000 ± 16.093	
AH	> 300.00 ± 0.000	0.001 [a] *	246.333 ± 45.621	0.001[b]*
FD-PRP	> 300.00 ± 0.000		269.667 ± 33.946	
AH + FD-PRP	> 300.00 ± 0.000		130.667 ± 14.571	

*significance p <0.05. [a]ANOVA test on the 5 groups on day 3rd. [b]ANOVA test on the 5 groups on day 14th.

Table II - Differences of the number of fibroblast on days 3 and 14.

Group	Number of fibroblasts			
	Day 3		Day 14	
	$\bar{x}$ ± SD	p value	$\bar{x}$ ± SD	p value
Control (-)	96.067 ± 6.379		119.867 ± 10,504	
Control (+)	108.467 ± 4.051		86.200 ±7.597	
AH	112.000 ± 2.029	0.025 [a] *	53.067 ± 9.453	0.001[b] *
FD-PRP	89.800 ±33,950		55.200 ±6.349	
AH + FD-PRP	180.267 ± 28.759		43.67 ±3.402	

*significance p <0.05. [a]Kruskall Wallis test on the 5 groups on the 3rd day. [b]ANOVA test on the 5 groups on day 14th.

Table III - Differences of PDGF-BB expression on days 3 and 14.

Group	PDGF-BB Expression			
	Day 3		Day 14	
	$\bar{x}$ ± SD	p value	$\bar{x}$ ± SD	p value
Control (-)	2,000 ± 0.000		3.330 ±0.577	
Control (+)	3,000 ± 0.000		3.000 ± 0.000	
AH	3.670 ±0.577	0.014 [a] *	2.000 ± 0.000	0.030 [b] *
FD-PRP	4.000 ± 0.000		3.000 ± 0.000	
AH + FD-PRP	4.000 ± 0.000		2.330 ±0.577	

*significance p <0.05. [a]Kruskall Wallis test on the 5 groups on the 3rd day. [b]Kruskall Wallis test on the 5 groups on the 14th day.

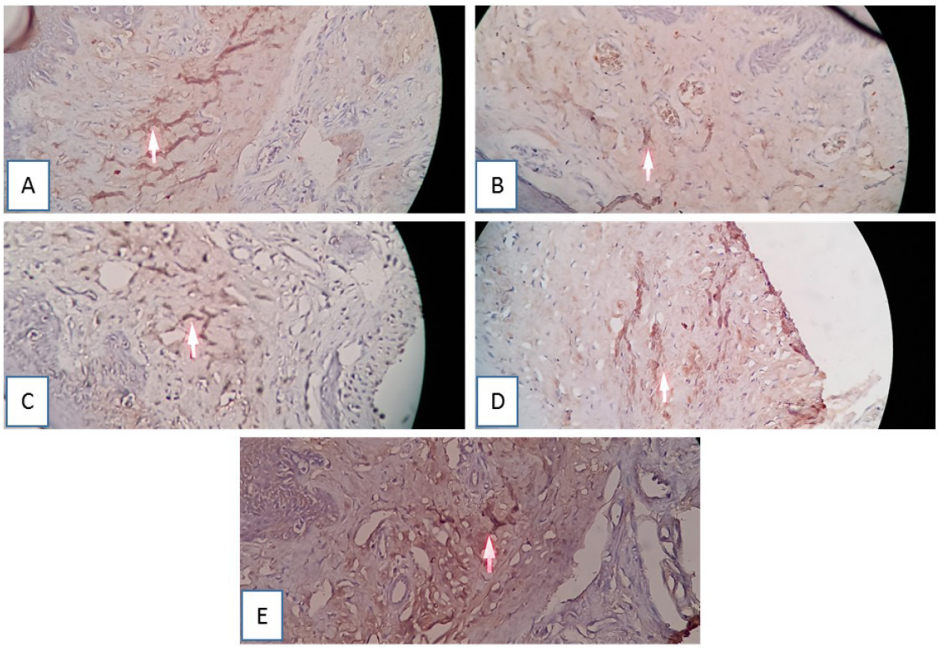

Figure 1 - Immunohistochemical features of PDGF-BB day 3. A. Negative control group, B. Positive control group, C. AH group, D. FD-PRP group, E. AH and FD-PRP combination group (magnification 400x).

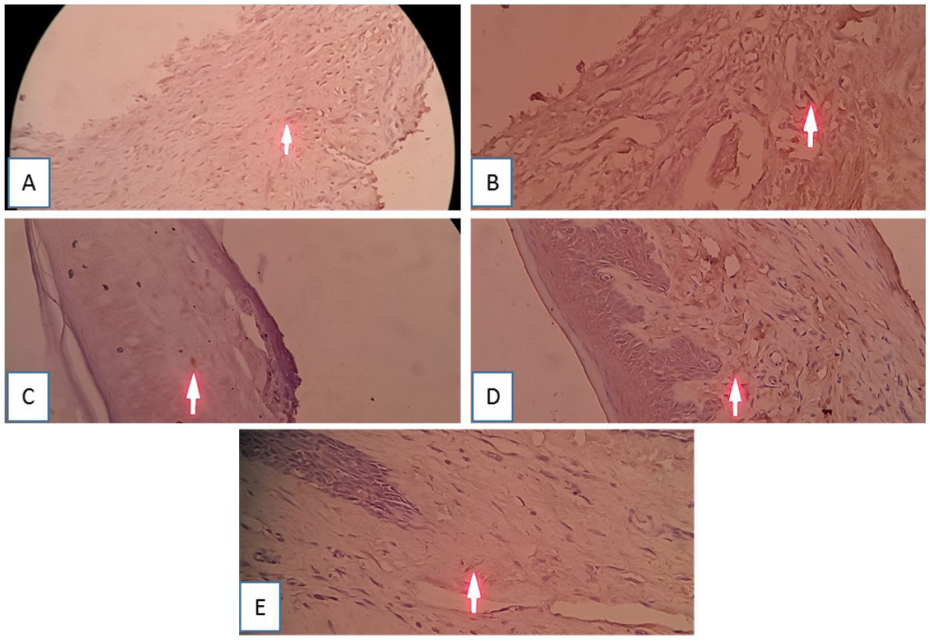

Figure 2 - Immunohistochemical features of PDGF-BB day 14. A. Negative control group, B. Positive control group, C. AH group, D. FD-PRP group, E. AH and FD-PRP combination group (magnification 400x).

Table IV - Differences of BoP on days 3 and 14.

Group	Bleeding on probing (BoP)			
	Day 3		Day 14	
	$\bar{x}$ ± SD	p value	$\bar{x}$ ± SD	p value
Control (-)	1,000 ± 0.000		1,000 ± 0.000	
Control (+)	1,000 ± 0.000		1,000 ± 0.000	
AH	0.670 ± 0.577	0.348 [a]	0.000 ± 0.000	0.007 [b] *
FD-PRP	0.330 ± 0.577		0.000 ± 0.000	
AH + FD-PRP	0.670 ± 0.577		0.000 ± 0.000	

*significance p <0.05. [a]Kruskal Wallis test on 5 groups on day 3. [b]Kruskal Wallis test on the 5 groups on the 14th day.

DISCUSSION

This research is an experimental study with a pre and post-test only group design. This design was chosen to sensitize research subjects who might give a different response compared to without the pre-test [12]. Wistar rats were selected as research samples because they are an ideal standard for experimental animal studies that are representative of mammals [13]. Murdiastuti et al. [14] stated that the content of growth factors in the collagen-activated FD-PRP had higher PDGF levels than conventional platelet rich plasma.

Researchers in this study proved the effectiveness of FD-PRP in combination with HA in the healing of periodontal surgical wounds in this article. Researchers found a decrease in the level of *P. gingivalis* on day three and day 14 in all treatment groups (Table I). This proves that FD-PRP and HA have antimicrobial properties against *P. gingivalis*. This is in accordance with the research of Chen et al. [15] who proved that hyaluronic acid has an antimicrobial effect against *P. gingivalis*. In another study, Pham et al. [16] also stated that platelet rich plasma has antimicrobial properties against *P. gingivalis*. Researchers found FD-PRP in combination with HA to have antimicrobial properties against *P. gingivalis* compared to FD-PRP or HA alone.

Based on histological examination in this study, it was found that on the third day there was an increase in the number of fibroblasts in the treatment group, while on the fourteenth day there was a decrease in the number of fibroblasts in the treatment group compared to the control group (Table II). This is important in wound healing, because in the early stages of wound healing, fibroblasts play an important role in stimulating proliferation, migration, cell differentiation and controlling protein synthesis and degradation [2]. However, at resolution of the healing phase, the number of fibroblast cells will decrease due to cell apoptosis [17]. In the treatment group, an increase in the number of fibroblasts compared to the control group will accelerate the healing process. This is evidenced by a greater decrease in the treatment group on the fourteenth day, which indicates the resolution of the healing phase which proceeds earlier than in the control group. Combination FD-PRP and HA group had the best histopathological test results in the form of the number of fibroblast cells compared to other groups.

Based on the immunohistochemical review of this study, it was found that the expression of PDGF-BB was higher in the treatment group compared to the control group on the third day (Figure 1). Increased expression of growth factors such as PDGF-BB can accelerate wound healing. Verma et al. [18] who stated that the application of autologous PRP can accelerate soft tissue healing and bone regeneration by increasing PDGF-BB. Lynch et al. [19] stated that increasing PDGF in the wound area can increase cell density in connective tissue. In addition, there is the formation of thicker connective tissue during the healing process. In addition, Komatsu et al. [20] stated that PDGF-BB enhances periodontal ligament regeneration by decreasing osteocalcin cells and increasing the proliferation and migration of fibroblast cells periodontium. Nevins et al. [21] stated that PDGF-BB can provide clinically significant attachment enhancement in the treatment of moderate to severe two and three-walled infrabony periodontal defects. Anusaksathien et al. [22] stated that maintaining PDGF levels in the third week after healing can inhibit growth and biomineralization. This indicates that continuous administration of PDGF can slow cementoblast-induced mineral formation.

Based on the result of this study, it was found that the BOP value in the treatment group was lower than the control group, both on the third day and on the fourteenth day (Table IV).

On the fourteenth day, all treatment groups had a BOP value of 0, which indicated an inflammation-free surgical area. Abdul Ameer et al. [23] stated that the application of PRP in the periodontal pocket has an anti-inflammatory effect that can accelerate the healing process. This will be seen in the improvement of periodontal clinical parameters, such as a decrease in plaque index, gingival index, BOP and clinical attachment level. Vera et al. [24] stated that hyaluronic acid can have an indirect effect on inflammation, stabilize granulation tissue and prevent the release of protease enzymes from inflammatory cells that cause inflammation can break down extracellular matrix proteins during the healing process. This research has limitations, including the need to find the best dose selection in the use of freeze-dried platelet-rich plasma to support the stability and durability of the material combined with FDPRP and hyaluronic acid.

CONCLUSION

The combination of FD-PRP and HA has accelerated the healing of periodontal surgical wounds.

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Author's Contributions

PW, DS, MA, B, RS: Researcher, Critically Revised the Manuscript. PW: Writing, Analysis, Interpretation. PW, DS, MA: Conception and Data Design. B, RS: Performed the Experiments.

Conflict of Interest

The authors certify that they have no commercial or associative interest representing a conflict of interest in connection with the manuscript.

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Regulatory Statement

Before the commencement of research, the ethical approval for the current study was obtained from the institutional ethical committee. The approval code for this study is No. 0350/KEPH-FMIPA/2021.

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Pitu Wulandari
(Corresponding address)
Universitas Sumatera Utara, Faculty of Dentistry,
Department of Periodontics, Medan, Indonesia.
Email: pitu.wulandari@usu.ac.id

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