Bacteria inhibition of platelet concentrates with and without pre-centrifugation metronidazole incorporation on *aggregatibacter actinomycetemcomitans*

Adiguna Putra Walianto, Sri Pramestri Lastianny**, Hendrawati***

** Clinical Dentistry Program, Faculty of Dentistry, Universitas Gadjah Mada*
*** Department of Periodontics, Faculty of Dentistry, Universitas Gadjah Mada*

Correspondence: adiguna_pw@mail.ugm.ac.id

Received 16 March 2022; 1st revision 14 June 2022; 2nd revision 17 Augustus 2022; Accepted 14 September 2022; Published online 28 December 2022

Keywords:

Platelet rich fibrin;injectable platelet rich fibrin; antibiotic; pre-centrifugation metronidazole incorporation; inhibition

ABSTRACT

Background: *Local administration of antibiotics was one of many approaches for the management of localized periodontal infection with the advantages of smaller total dosage delivered into the pocket, avoiding systemic antibiotic side effects, and increasing the exposure of target microorganism to higher concentration. Incorporating antibiotics into platelet concentrates can be considered for local antibiotic administration.*
Method: *The sample in this study used Aggregatibacter actinomycetemcomitans which was divided into 4 partitions on each plate. Each plate was applied with Platelets Rich Fibrin (PRF) and Injectable Platelet rich fibrin (I-PRF) incorporated with metronidazole pre-centrifugation and two negative controls (PRF and I-PRF without metronidazole). The observation was performed after 1 day and 7 days of incubation by measuring inhibition zone diameter using vernier caliper. The data were analyzed using two-way ANOVA followed by post-hoc LSD.*
Result: *I-PRF with metronidazole had the highest bacteria inhibition compared with other groups ($p<0,05$) on incubation day 1 and 7; I-PRF and PRF with metronidazole showed higher bacteria inhibition compared with I-PRF and PRF without metronidazole ($p<0,05$).*
Conclusion: *Aggregatibacter actinomycetemcomitans inhibition of PRF and I-PRF incorporated with metronidazole was greater compared to PRF and I-PRF without incorporating metronidazole, and A. actinomycetemcomitans inhibition of I-PRF incorporated with metronidazole was greater than PRF incorporated with metronidazole.*

doi: http://dx.doi.org/10.30659/odj.9.2.183-190
2460-4119 / 2354-5992 ©2022 National Research and Innovation Agency
This is an open access article under the CC BY-SA license (https://creativecommons.org/licenses/by-sa/4.0/)
Odonto : Dental Journal accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3200)
How to Cite: Walianto *et al*. Bacteria inhibition of platelet concentrates with and without pre-centrifugation metronidazole incorporation on aggregatibacter actinomycetemcomitans. Odonto: Dental Journal, v.9, n.2, p.183-190, December 2022.

INTRODUCTION

Periodontal disease is prevalent in both developed and developing countries and affects 20-50% of the world's population[1]. Periodontitis is characterized by inflammation mediated by host and related to microbes causing loss of periodontal attachment. Some species such as *Aggregatibacter (A.) actinomycetemcomitans* and *Porphyromonas gingivalis* are most well-known periodontal pathogen[2,3]. *A. actinomycetemcomitans* is assumed as main causative agent of aggressive periodontitis especially localized aggressive periodontitis and implicated on chronic periodontitis and high-risk non-oral infection[4].

Periodontal treatment aims to prevent further development of the disease. Periodontal treatment consists of non-surgical treatment, namely local and systemic pharmacotherapy such as the use of antiseptics, local and systemic antibiotics, and surgical treatment such as surgical resection of infected tissue for access to the root area and regenerative surgery[5]. One of the materials that can be used as adjunctive therapy in surgical treatment is platelet concentrate[6]. Platelets in platelet concentrates have an important role in hemostasis and wound healing, and growth factors produced by platelets are a source of healing cytokines. There are various types of platelet concentrates such as platelet rich plasma (PRP), platelet rich fibrin (PRF), and injectable platelet rich fibrin (I-PRF) which have regenerative properties. Platelet rich plasma uses additives that can cause the formation of antibodies against clotting factors V, XI, and thrombin, so PRF and I-PRF which are completely autologous can be the materials of choice[7,8].

The PRF fabrication protocol using high centrifugation forces causing a shift of the cell population to the bottom of the tube compared to the PRF layer, so I-PRF protocol which is based on the concept that slower and shorter centrifugation cycles result in the presence of higher regenerative cells with higher concentrations of growth factors and slower centrifugation rate indicates a higher number of cells including leukocytes before fibrin clot formation[9,10,11]. There is a study showing that I-PRF has a higher platelet count compared to PRP and PRF[12].

In addition to the known regenerative properties, platelet concentrates also have antimicrobial properties, but their antimicrobial properties are limited compared to antibiotics[7], so the addition of antibiotics to platelet concentrates can be considered. Local administration of antibiotics was one of many approaches for management of localized periodontal infection with the advantages of smaller total dosage delivered into the pocket, avoiding systemic antibiotic side effects, and increasing the exposure of target microorganism to higher concentration, thereby providing a medication with a higher therapeutic level[13], thus incorporating antibiotics into platelet concentrates can be considered for local antibiotic administration. One of many approaches to adding antibiotics into platelet concentrate is by adding antibiotics before the blood tube is centrifuged during the preparation of platelet concentrate or called pre-centrifugation.

Metronidazole is a broad-spectrum antibiotic against protozoa and anaerobic bacteria which is one of the antibiotics of choice in the treatment of periodontal disease. The rationale for using metronidazole is the specificity of the drug against anaerobic bacteria and preventing bacteria resistance[14]. Metronidazole can be the antibiotic of choice because of its effectiveness in the treatment of acute ulcerative gingivitis and has a narrow bactericidal spectrum so as to minimize the

risk of secondary opportunistic infections such as candidiasis and can be used as a systemic antibiotic drug in both chronic periodontitis and aggressive periodontitis[15]. Metronidazole has been used to treat periodontal disease in a systemic and local (subgingival) application in the form of a gel, and is the gold standard that is often used for the treatment of periodontal disease[16,17].

METHODS

This study was experimental laboratory study and passed ethical clearance with letter 0025/KKEP/FKG-UGM/EC/2022. The sample of this study was *A. actinomycetemcomitans*.

PRF with metronidazole incorporation was prepared by inserting 10 ml of blood sample from a volunteer into a glass tube, inserting 0.5 ml of metronidazole into the glass tube and then centrifuged at 2.700 rpm (maximum rcf 735 g) for 12 minutes. Fibrin clot in the center of the centrifuged blood was collected using sterile tweezer and separated from the red blood cell base using sterile scissor. Platelet rich fibrin without metronidazole incorporation was prepared as a negative control[18].

I-PRF with metronidazole incorporation was prepared by inserting 10 ml of blood sample from a volunteer into a plastic tube, inserting 0.5 ml of metronidazole into the plastic tube and then centrifuged at 700 rpm for 3 minutes. Fibrin fluid at the top of the centrifuged blood was collected using an injection syringe. Injectable platelet rich fibrin without metronidazole incorporation was prepared as a negative control[7,11].

The bacterial agar plate was partitioned into 4 parts for each platelet concentrate form. In each partition a punch with diameter of 4 mm is made in the center of the partition area. PRF was cut evenly with scissors according to the required number of samples and placed on the partition plate that has been punched ± 0.1 ml of injectable platelet rich fibrin was placed directly and evenly on the agar plate partition that has been punched. Bacterial agar plates that have been given a sample of platelet concentrate were marked according to the sample applied, closed, and put in a desiccator and incubated under anaerobic conditions at 37^{o}C for 1 day and 7 days according to group division based on incubation time[7,11,12,18,19, 20].

Sample data were collected by measuring and recording the diameter of the inhibition zone of the colony-forming unit on the agar plate using a vernier caliper (Tricle Brand) in millimeters, measured by measuring the vertical, horizontal, and diagonal diameters. The measurement results were the average of the three measurements [18,21.22.23].

The statistical test used was the two-way ANOVA test, followed by Least Significance Difference (LSD) test.

RESULTS

The results of antibacterial testing were expressed in terms of inhibition in the form of the diameter of the inhibition zone. The difference in the mean diameter of the inhibition zone at 1 day and 7 days of incubation was presented in Table 1.

Table 1. Mean diameter of the inhibition zone

Treatment Group		Number of Samples	Inhibition Zone Diameter (mm)
Test Material	**Incubation Duration**		
PRF	1 Day	7	0.00 ± 0.00
	7 Days	7	5.53 ± 0.23

PRF with Metronidazole Incorporation	1 Day	7	11.54 ± 0.36
	7 Days	7	11.91 ± 0.3
I-PRF	1 Day	7	6.12 ± 0.25
	7 Days	7	6.88 ± 0.44
I-PRF with Metronidazole Incorporation	1 Day	7	13.8 ± 0.39
	7 Days	7	14.09 ± 0.32

Based on Table 1 the mean diameter of the inhibition zone increased in each group at 7 days of incubation. In the treatment group with incubation period of 1 day and 7 days, the diameter of the inhibition zone from the narrowest to the widest were the PRF group, I-PRF, PRF with metronidazole incorporation and the widest was I-PRF with metronidazole incorporation. The difference in the mean diameter of the inhibition zone was presented in Figure 1.

Based on the results of normality test using Shapiro-Wilk test, mean diameter of the inhibition zone in each group were normally distributed as indicated by a significance value greater than 0.05 ($p>0.05$). Based on the results of the homogeneity test using Levene's test, mean diameter of the inhibition zone in each group had the same variance, which were indicated by a significance value 0.378 ($p>0.05$). These results indicate that the data were homogeneous.

Based on the results of the two-way ANOVA test (Table 2), test material, incubation duration, and the interaction between the test material and incubation duration have a significant effect on the inhibition of bacterial growth with a significance value of 0.000 ($p<0.05$). To determine the differences between groups of test materials and incubation time, further analysis was carried out using the LSD test.

Table 2. Two-way ANOVA test results of the zone of inhibition at incubation day 1 and 7

Dependent Variable: Inhibition of Bacterial Growth	
Independent Variable	*p* (Significance)
Test Material	0,000
Incubation Duration	0,000
Test Material*Incubation Duration	0,000

DISCUSSION

The results of the study on the antibacterial properties of PRF, I-PRF, PRF with metronidazole incorporation and I-PRF with metronidazole incorporation based on the incubation duration of 1 day and 7 days showed significant differences in all groups, with group I-PRF with metronidazole incorporation having the widest inhibition zone diameter, followed by PRF with metronidazole incorporation, I-PRF, and PRF. Platelet concentrates have antibacterial properties by various hypothesized mechanisms, namely: production of oxygen metabolites such as superoxide, hydrogen peroxide, and hydroxyl free radicals that were cytotoxic; binding, aggregation,

and internalization of microorganisms thereby increasing the clearance of pathogens from the bloodstream; participates in the cytotoxicity function of antibody-dependent cells to kill protozoan pathogens; and platelets releasing antimicrobial peptides that were potent. Activated platelets secrete platelet microbicidal protein (PMP) which had antibacterial activity by contacting with bacterial membranes, changing membrane permeability, entering bacterial cells, and inhibiting the synthesis of large molecules [24,25,26].

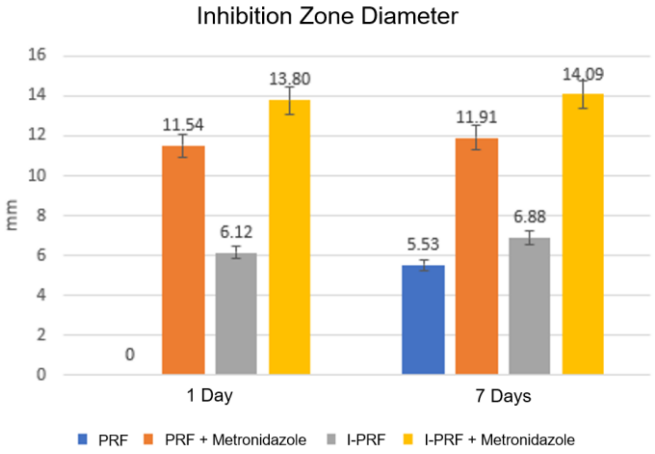

Figure 1. Graph of mean inhibition zone diameter

Injectable platelet rich fibrin with metronidazole incorporation group had the widest inhibition zone diameter and this result was supported by the study of Karde *et al.* (Year?)[12] that stated I-PRF showed the maximum zone of inhibition in the oral microflora compared to other platelet concentrates (PRF and PRP). This could be due to the PRF fabrication protocol using high centrifugation forces causing a shift of the cell population to the bottom of the tube compared to the PRF layer so I-PRF protocol which was based on the concept that slower and shorter centrifugation cycles result in the presence of higher regenerative cells with higher concentrations of growth factors and slower centrifugation rate indicates a higher number of cells including leukocytes before fibrin clot formation[9,10,11,12].

Platelet concentrates with pre-centrifugation metronidazole incorporation had wider inhibition zone than the without group at 1 day and 7 days of incubation. This result was supported by the study of Polak *et al*. (year?)[18] which stated that PRF incorporated with antibiotics prior to centrifugation showed significant antibacterial activity compared to PRF incorporated with saline. Fibrin was generally described acting as 'glue' for cells, growth factors and various other biomaterials such as antibiotics[11]. Metronidazole works by passing through the target cell membrane by passive diffusion then its nitro group was reduced to nitro radicals by ferredoxin or flavodoxine. The reduction of nitro groups by anaerobic organisms was responsible for its cytotoxic effects to the bacteria and antimicrobial[27].

Inhibition zone diameter of platelet concentrates with and without metronidazole

incorporation at incubation duration of 7 days was wider than at inbutaion duration of 1 day with a significant difference except that in I-PRF with metronidazole incorporation group the difference was not significant. This could be due to the fact that the fibrin matrix of PRF has a large number of platelets and leukocyte cytokines and these components were released gradually over time (7-11 days) due to the breakdown of the fibrin network and I-PRF releases growth factors slowly over a period of 10-14 days[7,11].

Inhibition zone diameter of I-PRF group was smaller than PRF with metronidazole incorporation group at 1 day and 7 days of incubation. This result was supported by the study of Jafarzadeh *et al*. (year?) [28] which stated that inhibition zone diameter of positive control antibiotics (penicillin and gentamicin) was wider than platelet concentrate PRP against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus epidermis*, *Staphylococcus aureus*, and *Micrococcus luteus* bacteria. This was because platelet concentrates have antimicrobial properties, but its properties were limited when compared to antibiotics[7].

I-PRF with pre-centrifugation metronidazole incorporation group with incubation duration of 7 days did not show a significant difference compared with incubation duration of 1 day. This could be due to the large release of metronidazole from I-PRF since 1 day of incubation and remained stable up to 7 days of incubation in accordance with the study of Rafiee *et al.* (year?)[29] which combined I-PRF with a mixture of three antibiotics (metronidazole, ciprofloxacin and minocycline) and observed up to 28 days showed that large releases of antibiotics occurred in the first 24 hours. Study conducted by Kamocki *et al.* (year?)[30] regarding Polydioxanone-based fibrous scaffold containing metronidazole and ciprofloxacin which showed a large release of both antibiotics in the first 24 hours followed by a continuous release for 14 days. I-PRF was a platelet concentrate that can be combined with various biomaterials easily to improve the properties of the biomaterial [29,31].

PRF group with an incubation duration of 1 day did not show a bacterial inhibition zone, while the I-PRF group with an incubation period of 1 day showed an incubation inhibition zone. These results contradicted with the study of Karde *et al.* (year?)[12] which stated that there was an inhibitory zone diameter on PRF and I-PRF against oral bacteria that were aerobically incubated for 48 hours. The results also contradicted with the study of Kour *et al.* (year?)[7] which stated that there was an inhibitory zone diameter on PRF and I-PRF against *A. actinomycetemcomitans* bacteria which were incubated anaerobically for 1 day. The results was supported by the study of Badade *et al.* (year?)[24] which stated that PRF did not show an inhibition zone against *A. actinomycetemcomitans* bacteria which were incubated anaerobically for 48 hours. This could be because PRF contains lower concentrations of platelets and leukocytes when compared to other platelet concentrates. Standard PRF had dense fibrin clots with minimal interfibrous space, fewer cells and less uniformity when compared to advanced platelet rich fibrin (A-PRF) which prepared with a slower and shorter centrifugation protocol. Larger interfibrous spaces result in greater accumulation of cells and their continued release. PRF was an autologous fibrin matrix that has a large number of platelets and leukocyte cytokines intrinsically embedded during centrifugation causing its release to occur gradually over time (7-11 days) when the fibrin was destroyed, so that during the first 48 hours after PRF was fabricated it might not show an antimicrobial effect yet[7,32,33].

CONCLUSION

Bacterial inhibition of platelet concentrates with pre-centrifugation metronidazole incorporation on the growth of *A. actinomycetemcomitans* bacteria was greater than platelet concentrates without pre-centrifugation metronidazole incorporation.

REFERENCES

1. Nazir MA. *Prevalence of periodontal disease, its association with systemic diseases and prevention*. Int J Health Sci (Qassim), 2017;11(2): 72-80
2. da Silva-Boghossian CM, do Souto RM, Luiz RR, Colombo APV. *Association of red complex, A. actinomycetemcomitans and non-oral bacteria with periodontal disease*. Arch Oral Biol, 2011;56(9): 899-906
3. Tonetti MS, Greenwell H, Kornman KS. *Staging and grading of periodontitis: Framework and proposal of a new classification and case definition*. J Periodontol, 2018;89(Suppl 1): S159-S172
4. Åberg CH, Kelk P, Johansson *A. Aggregatibacter actinomycetemcomitans: Virulence of its leukotoxin and association with aggressive periodontitis*. Virulence, 2015;6(3): 188-195
5. Graziani F, Karapetsa D, Alonso B, Herrera D. *Nonsurgical and surgical treatment of periodontitis: how many options for one disease?*. Periodontol 2000, 2017;75(1): 152-188
6. Marenzi G, Qorri ME, Sammartino P, Rusciano F, Gasparro R. *Platelet Concentrates in Oral Surgery: Indications and Limits. A Literature Review*. Curr Dent, 2019;1(1): 12-22
7. Kour P, Pudakalkatti PS, Vas AM, Das S, Padmanabhan S. *Comparative Evaluation of Antimicrobial Efficacy of Platelet-rich Plasma, Platelet-rich Fibrin, and Injectable Platelet-rich Fibrin on the Standard Strains of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans*. Contemp Clin Dent, 2018;9(Suppl 2): S325-S330
8. Varshney S, Dwivedi A, Pandey V. *Antimicrobial effects of various platelet rich concentrates-vibes from in-vitro studies-a systematic review*. J Oral Biol Craniofac Res, 2019;9(4): 299-305
9. Choukroun J, Ghanaati S. *Reduction of relative centrifugation force within injectable platelet-rich-fibrin (PRF) concentrates advances patients' own inflammatory cells, platelets and growth factors: the first introduction to the low speed centrifugation concept*. Eur J Trauma Emerg Surg, 2018;44(1): 87-95
10. Fujioka-Kobayashi M, Miron RJ, Hernandez M, Kandalam U, Zhang Y, Choukroun J. *Optimized Platelet-Rich Fibrin With the Low-Speed Concept: Growth Factor Release, Biocompability, and Cellular Response*. J Periodontol, 2017;88(1): 112-121
11. Miron RJ, Zhang Y. *Autologous liquid platelet rich fibrin: A novel drug delivery system*. Acta Biomater, 2018;75(2018): 35-51
12. Karde PA, Sethi KS, Mahale SA, Khedkar SU, Patil AG, Joshi C.P. *Comparative evaluation of platelet count and antimicrobial efficacy of injectable platelet-rich fibrin with other platelet concentrates*: An *in vitro* study. J Indian Soc Periodontol, 2017;21(2): 97-101
13. Klokkevold PR, 2019, *Treatment of Aggressive and Atypical Forms of Periodontitis*, In Newman MG, Takei HH, Klokkevold PR, Carranza FA, (Ed), Newman and Carranza's Clinical Periodontology, 13th Edition, Elsevier, Philadelphia, 480-1, 486
14. Pejčić A, Kesić L, Obradović R, Mirković D. *Antibiotics in the Management of Periodontal Disease*, Acta Facultatis Medicae Naissensis, 2010;27(2): 85-92
15. Kapoor A, Malhotra R, Grover V, Grover D. *Systemic antibiotic therapy in periodontics*. Dent Res J (Isfahan), 2012;9(5): 505-515
16. Ciancio SG, Mariotti AJ, 2019, *Systemic Anti-infective Therapy for Periodontal Diseases*, In Newman MG, Takei HH, Klokkevold PR, Carranza FA, (Ed), Newman and Carranza's Clinical Periodontology, 13th Edition, Elsevier, Philadelphia, 556, 559
17. Setiawan A, Lastianny SP, Herawati D. *Efektivitas Aplikasi Madu Murni Terhadap Penyembuhan Jaringan Periodontal pada Perawatan Periodontitis Penderita Hipertensi*. J Ked Gi, 2013;4(4): 228-235
18. Polak D, Clemer-Shamai N, Shapira L. *Incorporating antibiotics into platelet-rich fibrin: A novel antibiotics slow-release biological device*. J Clin Periodontol, 2019;46(2): 241-247
19. Febi QN, 2020, *Uji Antibakteri dan Viskositas Gel Flavonoid Daun Tembakau Kasturi terhadap Pertumbuhan Bakteri Aggregatibacter actinomycetemcomitans*, Skripsi, Universitas Jember, Jember
20. Maghsoudi O, Ranjbar R, Mirjalili SH, Fasihi-Ramandi M. *Inhibitory Activities of Platelet-Rich and Platelet-Poor Plasma on the Growth of Pathogenic Bacteria*. Iran J Pathol, 2017;12(1): 79-87

21. Bachtiar SY, Thahjaningsih W, Sianita N. *Pengaruh Ekstrak Alga Cokelat (Sargassum sp.) terhadap Pertumbuhan Bakteri Escherichia coli*. JMCS, 2012;1(1): 53-60
22. Husnani, Suhaimi, Puspasari H, Sari Y. *Uji Daya Hambat Kestrak Kental Daun Kratom (Mitragyna speciosa Korth) terhadap Bakteri Staphylococcus aureus sebagai Penyebab Jerawat*. Medical Sains, 2020;4(2): 95-100
23. Mozartha M, Silvia P, Sujatmiko B. *Perbandingan Aktivitas Antibakteri Ekstrak Curcuma zedoaria dan Bahan Irigasi Natrium Hipoklorit 2.5% terhadap Enterococcus faecalis*. JMKG, 2019;8(1): 22-29
24. Badade PS, Mahale SA, Panjwani AA, Vaidya, PD, Warang AD. *Antimicrobial effect of platelet-rich plasma and platelet-rich fibrin*. Indian J Dent Res, 2016;27(3): 300-304
25. Joshi CP, Patil AG, Karde PA, Khedkar SU, Arunkum S. *Autologous Platelet Rich Fibrin as a potential antiperiopathogenic agent: An in-vitro study*. IP Int J Periodontol Implantol, 2016;1(2): 50-54
26. Yeaman MR. *The role of platelets in antimicrobial host defense*. Clin Infect Dis, 1997;25(5): 951-958
27. Ceruleos AH, Romero-Quezada LC, Ledezma JCR, Contreras LL. *Therapeutic uses of metronidazole and its side effects: an update*. Eur Rev Med Pharmacol Sci, 2019;23(1): 397-401
28. Jafarzadeh E, Pourmokhtar M, Tavili S. *In Vitro Evaluation of the Anti-bacterial Effect of Human Platelet Concentrate*. IJBC, 2016;8(1): 5-8
29. Rafiee A, Memarpour M, Taghvamanesh S, Karami F, Karami S, Morowvat MH. *Drug Delivery Assessment of a Novel Triple Antibiotic-Eluting Injectable Platelet-Rich Fibrin Scaffold: An In Vitro Study,* Curr Pharm Biotechnol. 2021;22(3): 380-388
30. Kamocki K, Nör JE, Bottino MC. *Dental pulp stem cell responses to novel antibioric-containing scaffolds for regenerative endodontics*. Int Endod J, 2015;48(12): 1147-1156
31. Egle K, Salma I, Dubnika A. *From Blood to Regenerative Tissue: How Autologous Platelet-Rich Fibrin Can Be Combined with Oher Materials to Ensure Controlled Drug and Growth Factor Release*. Int J Mol Sci, 2021;22(21): 11553(1-18)
32. Ghanaati S, Booms P, Orlowska A, Kubesch A, Lorenz J, Rutkowski J, Landes C, Sader R, Kirkpatrick C, Choukroun J. *Advanced platelet-rich fibrin: a new concept for cell-based tissue engineering by means of inflammatory cells*. J Oral Implantol, 2014;40(6): 679-689
33. Ravi S, Santhanakrishnan M. *Mechanical, chemical, structural analysis and comparative release of PDGF-AA from L-PRF, A-PRF and T-PRF – an in vitro study*. Biomater Res, 2020;24: 16(1-10)