

Autologous Platelet Rich Fibrin as a potential antiperiopathogenic agent: An in-vitro study

Chaitanya Pradeep Joshi^{1,*}, Agraja Ganpat Patil², Prerna Ashok Karde³, Smita Uday Khedkar⁴, Swapna Arunkumar Mahale⁵, Nitin Hemchandra Dani⁶

^{1,2,3}PG Student, ⁵Professor & HOD, ⁶Ex-Professor & HOD, Dept. of Periodontics, ⁴Professor, Dept. of Microbiology, MGVS KBH Dental College & Hospital, Nasik

***Corresponding Author:**

Email: chaitanya.joshi87@gmail.com

Abstract

Background: Lately, hard and soft tissue regeneration property of autologous platelet concentrates is gaining a lot of popularity in dental and allied fields. Along with intrinsic regenerative properties, platelets are hypothesized to release certain antimicrobial peptides. Aim of present study is to evaluate antimicrobial activity of platelet-rich fibrin (PRF) preparation against periodontal pathogenic bacteria.

Methods: Subgingival plaque samples and blood samples from fifteen generalized chronic periodontitis patients were collected. Antimicrobial activity of PRF preparation against periodontal bacteria was assessed. Disc diffusion method and colorimetric analysis were employed to quantify the inhibitory effects.

Results: PRF demonstrated clear zone of inhibition in disc diffusion method and showed minimum amount of turbidity confirmed by colorimetric analysis. This clearly indicates possible bactericidal activity against gram-negative periodontopathic bacteria.

Conclusion: Apart from being excellent regenerative material, through its antibacterial effects, PRF can provide additional benefits at periodontal surgical sites.

Keywords: PRF, Periodontal pathogens, Antibacterial agent, Disc diffusion, Colorimetry

Introduction

Presently, platelet derived concentrates such as Platelet Rich Plasma (PRP) and Platelet Rich Fibrin (PRF) have gained a lot of popularity as hard and soft tissues regenerative material. Its regeneration potential is thought to stem from the fact that platelets store growth factors such as Platelet Derived Growth Factors (PDGF), Transforming Growth Factor (TGF- β), Endothelial Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF), Insulin like Growth Factor (IGF-1), Fibroblast Growth factor (FGF) and other active biomodifying peptides which are released at local desired site upon activation. Additionally, platelet-derived concentrates also impart anti-inflammatory properties as evident by reduction in postoperative pain and swelling.^{1,2,3}

Depending upon the technique employed, different platelet-derived preparations with different biological and cellular characteristics can be obtained. In literature they are classified into four main categories based on their leukocyte and fibrin content.⁴

- Pure platelet-rich plasma (P-PRP),
- Pure platelet-rich fibrin (P-PRF),
- Leukocyte- and platelet-rich plasma (L-PRP),
- Leukocyte- and platelet-rich fibrin (L-PRF).

Choukruon's PRF is one of most widely used platelet concentrate since its inception. The fact that it is an open-access technique which utilizes simple and bare minimal armamentarium makes it more practical, less time consuming and least expensive yet highly predictable.⁵

Apart from its excellent regenerative properties, studies have reported an antimicrobial effect of platelet concentrates against bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*.^{6,7} The mechanism of the antibacterial effect of platelet-derived preparations is not yet fully understood. It is hypothesized that platelets demonstrate antimicrobial activity by the following ways:

- They generate oxygen metabolites, including superoxide, hydrogen peroxide and hydroxyl free radicals.^{8,9,10}
- They bind, aggregate and internalize microorganisms, which enhances the clearance of pathogens from the bloodstream;
- Platelets release an array of potent antimicrobial peptides^{11,12}

Although PRF is used in variety of periodontal surgical procedures for its superior regenerative potential, its antibacterial activity against periodontopathic bacteria remains an unexplored territory. Hence the aim of the current in-vitro study was to evaluate the antibacterial property of PRF preparation against periodontal pathogenic bacteria.

Material and Methods

Participant Selection: Blood samples were collected from 15 generalised chronic periodontitis who reported to the Department of Periodontology at MGVS KBH Dental College and Hospital, Nashik from November 2015 to December 2015. All were systemically healthy males (Mean age 48 \pm 0.4 years), non-smokers, with no

history of infection or any antibiotic use for at least 3 months. None of them gave history of any anticoagulant or immunosuppressive therapy that might interfere with natural coagulation process. In all patients periodontal regeneration was attempted using autologous PRF as a regenerative biomaterial. Simultaneously an additional written informed consent was obtained from all the patients to procure a piece of PRF membrane and plaque sample as a part of current study. The study was approved by the institutional ethical committee and research strictly adhered to the guidelines of declaration of Helsinki.

Plaque sample collection: Subgingival plaque samples were collected by single examiner (CJ). Patients with at least three sites with probing depth \geq 6mm were selected for sample collection. Just before the periodontal surgical intervention, the sites were thoroughly isolated with cotton rolls and supragingival deposits were scaled with a sterile curette^c. No. 30 sterile paper point⁸ was inserted into the apex of each periodontal pocket and kept for approximately 20

seconds.¹³ Upon removal, paper points from each patient were pooled in a single test tube containing Robertson's cooked meat media (RCM)[#] for transportation to the Dept. of Microbiology. Same protocol was repeated for each patient. Patients' samples were grown in RCM for 4-6 hours in an incubator at 37°C. Development of turbidity confirmed viability of sample.

Preparation of PRF: As a part of surgical procedure, 5 ml of intravenous blood from antecubital site was collected in the plain sterile glass test tube without anticoagulant and centrifuged at 3000 rpm for 10 minutes.⁵ After centrifugation; PRF clot was removed from the test tube using sterile tweezers and separated from the RBC base using a scissor. PRF concentrate was laid down on saline dampened gauze piece and using a sterile agar punch two circular discs of PRF clot were obtained for the current study. (Fig. 1) Rest of the PRF clot was utilized to prepare membrane to be used for the surgical periodontal regeneration procedure.

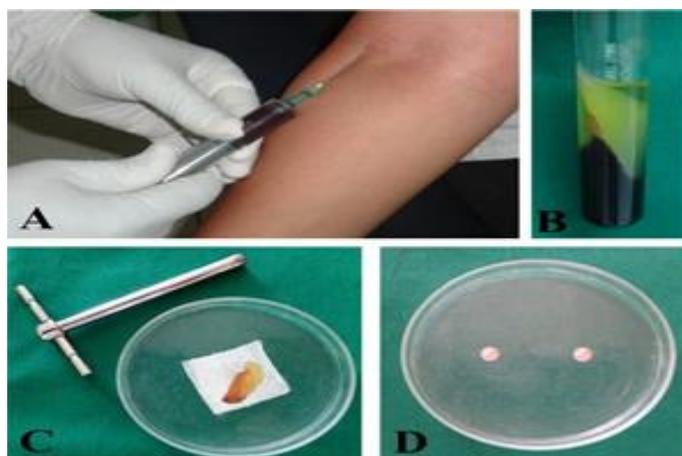


Fig. 1: A) Venous blood collection B) PRF clot obtained after centrifugation of blood at 3000 rpm for 10 min C) Prepared PRF clot and sterile agar punch D) Punched circular discs of PRF clot

Antimicrobial Activity of PRF membrane:

Colorimetric Analysis: Bacterial sample from each patient was inoculated in test tubes containing 5ml of Brain-Heart Infusion (BHI) broth and a disc of PRF. The test tube was then incubated at 37°C in an anaerobic chamber for 48 hours. Tests were performed in triplicates to assess veracity of the results. Sterile BHI broth containing tube served as a negative control. Additionally, test tubes with BHI broth along with patient's plaque sample without PRF and with metronidazole (5 µg) were used independently as controls for each individual. 5µg concentration of metronidazole was selected based on the fact that proven its MIC value against periodontopathic bacteria is 1µg/ml.¹⁴ Procedure was repeated for each patient's sample. Colorimeter* was calibrated using sterile BHI broth by setting it as zero optical density (OD). After 48 hours, growth of sample inoculated by plaque sample was estimated by measuring of OD of incubated bro that 530nm wavelength.

Agar Disc Diffusion Method: Simultaneously, patient's sample was spread on a sterile blood agar plate to obtain lawn culture. A second punched piece of PRF membrane was placed on inoculated agar plate. Metronidazole disc[#] was placed to confirm presence of anaerobic bacteria. Inoculated blood agar plates were then incubated in an anaerobic jar at 37° C for 48 hours. For all the patients procedure was repeated.

Microscopic analysis: Upon 48 hours incubated, sites immediately next to PRF membrane and metronidazole disc were selected for microbial for microbial analysis. Smears were prepared using sterile wire loop, fixed and gram staining was performed. Samples from all the patients were assessed in a similar fashion.

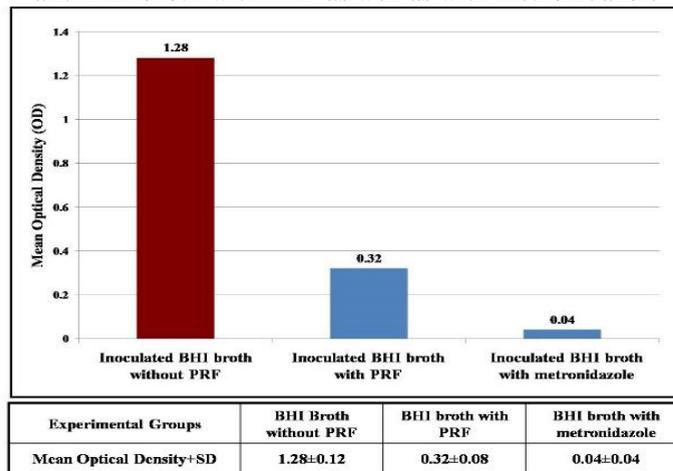
Results

Statistical Analysis: For colorimetric analysis, the statistical significance of the differences between all three groups was analyzed by one-way analysis of variance (ANOVA). Regarding results of disc diffusion method, comparison between zone of inhibition obtained around metronidazole and PRF groups was done using unpaired student’s t-test. P value <0.05 was considered significant. For statistical analysis ‘SPSS-9’ software was used.

Antimicrobial Activity of PRF:

Colorimetric Analysis: Colorimetric analysis by evaluating OD value is a common place procedure to estimate bacterial growth. In present study, mean OD value (0.32) of test sample (with PRF) was significantly less than the mean OD value (1.28) of positive control (without PRF) confirming inhibitory effects of PRF against periopathogenic bacteria (gram negative rod shaped bacteria).(Graph 1)

Graph 1: Comparison of mean optical density values of sample in BHI broth without PRF (positive control) and BHI broth with PRF as well as with metronidazole



Agar Disc Diffusion Method: Consistent clear zone of inhibition around metronidazole disc confirmed the presence of anaerobic bacteria in each sample.(Fig. 2) Mean zone of inhibition around metronidazole disc was 9.34±0.08 mm. For each test sample, PRF membrane also demonstrated a well demarcated zone of inhibition around it. A mean inhibition zone for PRF membrane was 6.45 ±0.05mm.(Graph 2) Difference between the mean values of zone of inhibition around both the groups reached statistical significance. These results indicate that the PRF disc also has a significant inhibitory effect on growth of periopathogens.

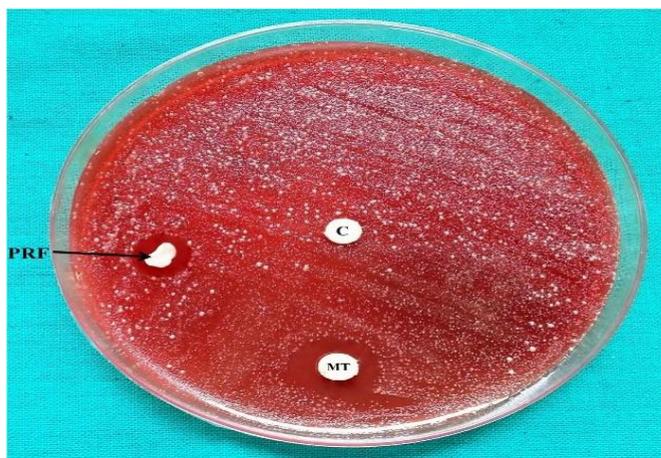
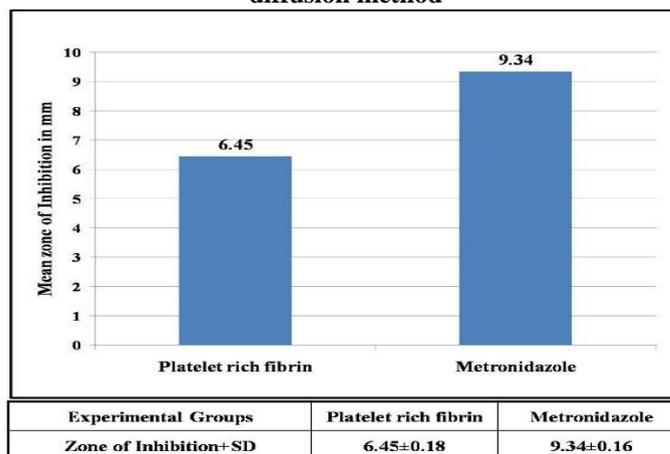


Fig. 2: Growth inhibition of periodontopathogens by PRF using disc diffusion method. MT=Metronidazole; C=Control

Graph 2: Comparison of mean zone of inhibition in millimetres by PRF and metronidazole using disc diffusion method

Microscopic analysis: Microscopic analysis revealed that there is substantial reduction in gram-negative rod shaped bacteria around metronidazole disc followed by PRF disc suggestive of pronounced bactericidal activity against likely periopathogenic bacteria.(Fig. 3)

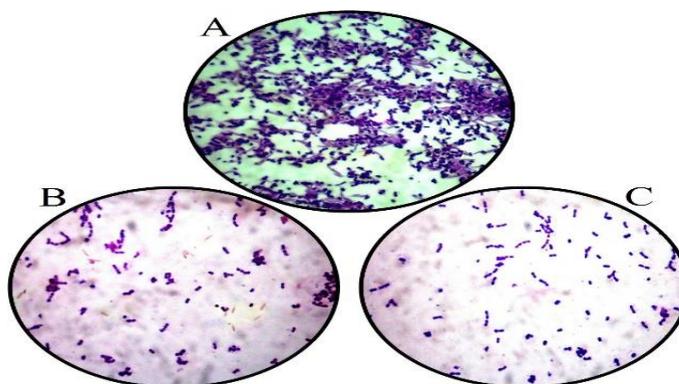


Fig. 3: A) Normal bacterial morphological profile of patient's plaque sample B) Substantial reduction of gram-negative rod shaped bacteria around PRF membrane C) Maximum reduction of gram-negative rod shaped around metronidazole disc

Discussion

Platelets release a group of cationic antimicrobial peptides (CAMPs) collectively known as platelet microbicidal proteins (PMPs) at site of endovascular damage or infection.¹² Although its precise mechanisms is still not clear, studies have demonstrated that PMPs depolarize, de-energize, and permeabilize microbial cell membranes and may subsequently lead to inhibition of macromolecular synthesis of target organisms.^{15,16} Several studies have also underlined the role of human platelets as a source of antimicrobial peptides namely platelet factor-4, RANTES, connective tissue activating peptide-3, platelet basic protein, thymosin beta-4, fibrinopeptide A and B, human beta defensin-3.^{6,10} Results of our study are in accordance with these studies in validating potential antimicrobial activity. The gram negative bacteria from patient samples were substantially reduced in numbers as demonstrated by both methods namely calorimetric and disc diffusion

method. Bacteria implicated as periodontopathic are proven to be primarily as gram negative rod shaped bacteria.¹⁷ Besides this platelets also aid in navigation of inflammatory cellular motility towards chemo attractants such as N-f-MetLeuPhe and complement C3a/C5a factors. Platelets are also known to be involved in production of known antimicrobial substances like superoxide ions, hydrogen peroxide and hydroxyl free radicals.¹²

Yeaman in 1997 suggested that direct interaction of platelets with microorganisms, participation in antibody-dependent cell cytotoxicity and engulfment by entrapped white blood cells within PRF could result in direct bacterial killing. Release of myeloperoxidase, activation of the antioxidant responsive elements and antigen-specific immune response have also been suggested.¹⁰ In another study by Drago L in 2013 using platelet concentrate in the form of P-PRP, it was found that P-PRP inhibited the growth of *E. fecalis*,

C.albicans, *S.agalactiae* and *S.oralis* but not against *P.aeruginosa*.¹⁸ All these studies indicate plausible antibacterial potency of platelet concentrates.

In the present study, we were clearly able to demonstrate the antimicrobial activity of PRF specifically against periodontopathic bacteria. These antimicrobial results are in accordance with observations of above stated studies. Possible intrinsic antibacterial property of PRF can definitely be utilized in reducing local site specific subgingival microbial bioburden and it can be an excellent alternative to conventional antimicrobial agents. Though metronidazole has more antimicrobial potency, PRF being an autologous preparation, is free from any side effects which we routinely encountered with other antimicrobial agents. Thus instead of conventional antimicrobial agents, antimicrobial potential of PRF can further be explored in controlling post-operative site specific infections.

Limitations

Identification of periodontopathogenic bacteria present in sample was not possible due to inaccessibility to such facility. It is an in-vitro study which is carried out in a static environment. Results might defer under dynamic oral conditions. Also the influence of patients' characteristics like sex, age, hematocrit, platelet count, etc on antimicrobial activity of PRF should also be investigated.

Conclusions

Autologous PRF is a simple and inexpensive technique used for the successful evidence based regeneration of lost periodontal tissues. Its antibacterial efficacy as demonstrated by present study will certainly add whole new meaning as well as dimension to its use. It will be an added benefit in reducing microbial load at periodontally infected sites. However this is being an in-vitro study, its results should further be evaluated and validated through more in-vivo studies.

Acknowledgement

The authors wish to thank Mr. Pradeep K. Joshi for his assistance.

Conflicts of interest

There are no conflicts of interest.

References

- Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, Gogly B: Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part III: leukocyte activation: new feature for platelet concentrates? *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2006;101:e51-55.
- El-Sharkawy H, Kantarci A, Deady J, Hasturk H, Liu H, Alshahat M, van Dyke TE: Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J Periodontol.* 2007;78:661-669.
- Del Fabbro M, Ceresoli V, Lolato A, Taschieri S: Effect of platelet concentrate on quality of life after periradicular surgery: a randomized clinical study. *J Endod.* 2012;38:733-739.
- Dohan Ebnfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol.*2009;27:158-167.
- Shah M, Deshpande N, Bharwani A, Nadig P, Doshi V, Dave D. Effectiveness of autologous platelet-rich fibrin in the treatment of intra-bony defects: A systematic review and meta-analysis. *J Indian Soc Periodontol.* 2014;18:698-704.
- Yuan T, Zhang CQ, Zeng BF. Treatment of chronic femoral osteomyelitis with platelet-rich plasma (PRP): A case report. *Transfus Apher Sci.* 2008;38:167-173.
- Anitua E, Alonso R, Girbau C, Aguirre JJ, Murozabal F, Orive G. Antibacterial effect of plasma rich in growth factors (PRGF-Endoret) against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. *Clin Exp Dermatol.* 2012;37:652-657.
- Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B et al. Thrombocidines, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. *J Biol Chem.* 2000;275:20374-20381.
- Wecksler BB, Nachman RL. Rabbit platelet bactericidal protein. *J Exp Med.* 1971;134: 1114-1130.
- Yeaman MR. The role of platelets in antimicrobial host defense. *Clin Infect Dis.*1997;25: 951-968.
- Klinger MH, Jelkmann W. Role of blood platelets in infection and inflammation. *J Interferon Cytokine Res.* 2002;22:913-922.
- Tang YQ, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun.* 2002;70: 6524-6533.
- Jervøe-Storm PM, Alahdab H, Koltzsch M, Rolf Fimmers, Søren Jepsen. Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. *J Periodontol* 2007;78:909-917.
- Eick S, Seltmann T, Pfister W. Efficacy of antibiotics to strains of periodontopathogenic bacteria within a single species biofilm - an in vitro study. *J Clin Periodontol.* 2004;31:376-383.
- Mercier RC, Rybak MJ, Bayer AS, Yeaman MR. Influence of platelets and platelet microbicidal protein susceptibility on the fate of *Staphylococcus aureus* in an in vitro model of infective endocarditis. *Infect Immun.* 2000;68:4699-4705.
- Tohidnezhad M, Varoga D, Podschun R, Wruck CJ, Seekamp A, Brandenburg LO et al. Thrombocytes are effectors of the innate immune system releasing human beta defensin-3. *Injury.* 2011;42:682-686.
- Sigmund S, Socransky and Anne D. Haffajee. Periodontal microbial ecology. *Periodontol* 2000 2005;38:135-87.
- Drago L, Bortolin M, Vassena C, Romao CL, Taschieri S, Del Fabbro M. Plasma Components and Platelet Activation Are Essential for the Antimicrobial Properties of Autologous Platelet –Rich Plasma: An In Vitro Study. *PLoS One.* 2014;18;9(9):e107813.