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Comparative Estimation of Antimicrobial Activity of Platelet-Rich Fibrin Incorporated with Metronidazole and Amoxicillin Against Porphyromonas Gingivalis and Fusobacterium Nucleatum – An Invitro Study

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Abstract:

Background and Objectives: *Surgical wound healing is often associated with a risk of infection. Despite strict disinfection measures, microorganisms may still invade and colonize the wound area, leading to tissue damage and delayed healing. Effective infection control is therefore essential for the success of any surgical procedure. In this context, the present in-vitro study aimed to evaluate the antimicrobial effect of Platelet-Rich Fibrin (PRF) incorporated with Metronidazole, Amoxicillin, and their combination against Porphyromonas gingivalis and Fusobacterium nucleatum.*

Materials and methods: *Blood samples were obtained from forty systemically healthy volunteers, and platelet-rich fibrin was prepared by adding 0.5 ml of antibiotics (Metronidazole 5 mg/ml, Amoxicillin 50 mg/ml, combination of Metronidazole 5 mg/ml and Amoxicillin 50 mg/ml) before centrifugation. The antibacterial activity of the prepared PRF was then evaluated using an antibiogram assay against standard strains of Porphyromonas gingivalis and Fusobacterium nucleatum.*

Results: *The zone of inhibition against Porphyromonas gingivalis and Fusobacterium nucleatum was significantly greater for combination of PRF with Metronidazole and Amoxicillin, followed by PRF with Amoxicillin, PRF with metronidazole, PRF alone.*

Conclusions: *Platelet-rich fibrin enriched with a combination of Metronidazole and Amoxicillin demonstrated a sustained antibacterial effect against Porphyromonas gingivalis and Fusobacterium nucleatum. This modified form of PRF may serve as a useful approach to minimize post-operative infections while retaining the natural healing benefits of PRF.*

Key words: *Platelet-rich fibrin, Amoxicillin, Metronidazole, periodontitis, local drug delivery*

I. INTRODUCTION

Periodontitis is an inflammatory condition affecting the supporting structures of the teeth. It is triggered by specific microorganisms or groups of microorganisms and leads to the gradual destruction of periodontal tissues, along with the development of pockets, gingival recession, or both [1].

Using checkerboard DNA–DNA hybridization, **Socransky et al.** identified five microbial complexes that are consistently present together in subgingival biofilms. Among these, the "red complex" is regarded as the most pathogenic. Apart from the red complex, several other bacterial combinations have also been linked to periodontitis. For example, *Porphyromonas gingivalis* (Pg) and *Aggregatibacter actinomycetemcomitans* (Aa), as well as *Fusobacterium nucleatum* (Fn), have been reported in multiple studies to play a significant role in periodontal disease progression [2].

The management of periodontitis typically involves mechanical debridement, periodontal surgery, and the use of local or systemic antibiotics. The primary goal of these therapies is to control infection, which is essential for proper wound healing and regeneration of periodontal tissues. However, surgical procedures always carry a risk of bacterial contamination, and in some cases, bacteria may persist and invade deeper tissues despite strict disinfection measures [3,4].

Recently, platelet concentrates have gained attention for their role in effectively managing the manifestations of periodontal diseases. Their regenerative potential and anti-inflammatory properties have been widely studied. The regenerative effect of platelet concentrates is believed to result from the release of various growth factors by the platelets [5,6].

Wound healing after surgery always carries a risk of infection. Despite strict disinfection protocols, microbes can still invade and colonize the underlying tissues, leading to tissue damage and delayed healing. Therefore, effective infection control is essential for the success of any surgical procedure. Although there is limited evidence supporting the use of peri- and post-operative systemic antibiotics in dental surgery, their potential adverse effects and the risk of promoting antibiotic-resistant bacteria make their routine use controversial [7,8].

In addition to their regenerative potential, platelet concentrates have been reported to exhibit antibacterial activity against *Staphylococcus aureus* [9], *Escherichia coli* [10], *Klebsiella pneumoniae* [11], and *Streptococcus oralis* [12], among other microorganisms. The leukocytes present in PRF are known to contribute to this antimicrobial effect[9]**. Cieslik-Bielecka et al. (2007)** demonstrated that platelet-rich gels lacked antibacterial properties; however, producing the gel 30 minutes after intravenous administration of amoxicillin and clavulanic acid (Augmentin) endowed it with strong antibacterial activity against *Enterococcus faecalis* [13]. Furthermore, **Miron and Zhang (2018)**, in an extensive review, discussed the potential of combining various bioactive materials with liquid PRF to create an advanced local delivery system for both small and large biomolecules [14]. Collectively, rather than administering systemic antibiotics to patients prior to PRF preparation, as suggested by **Cieslik-Bielecka et al. (2007)**, directly incorporating antibiotics into PRF may be a more practical approach. Nevertheless, to date, no method has provided clear evidence demonstrating the successful incorporation of antimicrobial agents into PRF.

Therefore, the aim of the present study was to develop a simple and practical method to impart antimicrobial properties to PRF and to provide in vitro evidence of its effectiveness. This modified PRF could offer additional benefits alongside its established healing properties and potentially reduce the reliance on systemic antibiotics in various oral surgical procedures.

II. MATERIALS AND METHODS

Forty systemically healthy volunteers were included in this randomized controlled trial to compare and evaluate the antimicrobial activity of Platelet-rich fibrin with Platelet-rich fibrin incorporated with Metronidazole, Amoxicillin and combination of Metronidazole and Amoxicillin against Porphyromonas gingivalis and Fusobacterium nucleatum.

The study subjects were recruited from the outpatient department of Periodontics at the College of Dental Sciences, Davangere, Karnataka. The study protocol was conducted in accordance with the guidelines of Rajiv Gandhi University of Health Sciences, Bangalore, and received approval from the local Ethics Committee of the College of Dental Sciences, Davangere, Karnataka, India. The study was carried out over a period of 1 to 1.5 years.

Patient selection criteria:

The patients were selected with the following inclusion and exclusion criteria from both the sexes.

Inclusion criteria:

- Patients with age group between 18-45 years.

Exclusion criteria:

- Patients under anti- platelet drugs.
- Patients with any blood disorders.
- Patients on antibiotics and steroids.
- Patients with any systemic conditions.
- Smokers and alcoholics
- Pregnant or lactating women

Sample size estimation

- 40 patients; determined based on the formula:

  Sample size(n) = $Z^2\sigma^2/e^2$

where,

Z is the Statistic value at 95% confidence interval (=1.96)

σ is the observed standard deviation (=0.16) [1]

e is the permissible error (=0.1)

Control group

- **Group 1**: 10 Platelet-rich fibrin clots without incorporation of antimicrobials.

Experimental group

- **Group 2**: 10 Platelet-rich fibrin clots with incorporation of METRONIDAZOLE 5mg/ml.
- **Group 3:** 10 Platelet-rich fibrin clots with incorporation of AMOXICILLIN 50mg/ml.
- **Group 4:** 10 Platelet-rich fibrin clots with incorporation of combination of METRONIDAZOLE 5mg/ml and AMOXICILLIN 50mg/ml.

Total samples = number of samples X number of Groups

= 10×4 = 40 samples

Therefore, Total Sample Size is 40 patients (10 samples in each group)

Armamentarium: (Fig 1)

1. Surgical/examination gloves
2. Kidney tray
3. Gauze pieces
4. Tweezer
5. Adson Toothed Forcep
6. Adson Toothless Forcep
7. GDC UNC-15 probe
8. Straight Scissor
9. Curved Scissor
10. DISPOVAN 5ml syringe
11. DISPOVAN 10ml syringe
12. Saline
13. Amoxicillin Vial
14. Metronidazole Vial
15. Test Tubes
16. Test tube Stand
17. Porphyromonas Gingivalis and Fusobacterium nucleatum inoculated agar plates

Fig.1 Armamentarium

Procedural steps:

I) Collection of Blood

- Blood samples were collected from forty healthy volunteers using 10 ml glass tubes without anticoagulants via venous puncture. (Fig 2)

II) Antibiotics incorporation into PRF

- Before centrifugation, antimicrobials were added to the freshly collected blood using a syringe at a volume of 0.5 ml. (Fig 3 & 4)
- The following antimicrobials and concentrations were tested:
  - (i) Metronidazole 5 mg/ml
  - (ii) Amoxicillin 50mg/ml
  - (iii) Combination of Metronidazole 5mg/ml and Amoxicillin 50mg/ml

III) PRF preparation

- The tubes were immediately centrifuged at 2,700 rpm for 12 minutes at room temperature using a fixed-angle centrifuge (Fig. 5).
- Following centrifugation, the platelet-rich fibrin clot, situated in the middle layer, was carefully retrieved with sterile tweezers and gently separated from the underlying red blood cell layer using sterile scissors (Fig. 6).
- Each volunteer yielded two PRF clots (Fig. 7).

IV) Anti-biogram assay

- The anti-biogram assay was performed immediately after PRF preparation on the standard strains of *Porphyromonas gingivalis* (Pg33277) and *Fusobacterium nucleatum* (Fn25586).
- The antimicrobial activity of the tested materials was assessed by measuring the diameter of the zones free of colony-forming units.
- The results were expressed as the mean of all measured diameters for each sample.

Fig 2.

Fig 3.

Fig 4.

(Figure 2, 3, 4: Phlebotomy; Addition of antibiotics prior to centrifugation; Before Centrifugation)

Fig 5.

Fig 6.

Fig 7.

(Figure 5, 6, 7: Centrifugation machine; After centrifugation; PRF clot formed after centrifugation)

Microbiological procedure:

Microorganisms used:

1. *Porphyromonas gingivalis* - ATCC 33277 (Strain)
2. *Fusobacterium nucleatum* - ATCC 25586 (Strain)

Microbial culturing:

Fig 8

Inoculation media for microbial culture was kept ready (Fig 8). *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were preserved in glycerol stocks at Dextrose Technologies Pvt. Ltd. Bangalore. The culture plates were placed in an anaerobic jar, and an anaerobic environment was established using a mixture of sodium borohydride, citric acid, and sodium bicarbonate in the presence of a palladium catalyst. Residual oxygen was removed using a suction apparatus, and the anaerobic conditions were continuously monitored with a pressure gauge attached to the jar. The plates were incubated at 37ºC for at least 72 hours prior to the study to ensure activation under anaerobic conditions. (Fig 9, 10)

Anti-biogram assay:

About 100µl of pre-cultured test organisms were spread onto the specific agar plates i.e., 5% Blood Agar plate for *Porphyromonas gingivalis* and Trypton Soya Agar plate for *Fusobacterium nucleatum*. Samples to be tested were placed on the bacterial plates and plates were incubated at 37ºC for 48 hours, under anaerobic conditions with 5% CO_2 and 95% Nitrogen for *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. After 48 hours zone of inhibition was measured using Vernier caliper and results were tabulated. (Fig 11)

Fig 9.

Fig 10.

Fig 11.

(Fig. 8, 9, 10, 11: Inoculation media for Porphyromonas gingivalis and Fusobacterium nucleatum; Bio Safety Cabinet for Inoculation Media; Anaerobic work station; Antibacterial Susceptibility Test using Disc Diffusion)

III. STATISTICS

Statistical analysis was done with SPSS software. Data comparison was done by applying specific statistical tests to find out the statistical significance of the results. Mean, standard deviation (SD), t value, F value and probability value were calculated.

Statistical analysis of zone of inhibition:

Repeated measure Analysis of Variance (ANOVA) test was used for intra group comparisons followed by Tukey's Post Hoc analysis.

Repeated measures ANOVA

- Repeated Measures ANOVA is a technique used to test the equality of means.
- F = Mean sum of squares between groups/Mean sum of squares within Groups

Unpaired t test or independent sample t test:

- The unpaired t method tests the null hypothesis that the population means related to two independent, random samples from an approximately normal distribution are equal **(Altman, 1991; Armitage and Berry, 1994).**
- When two separate sets of independent and identically distributed samples are obtained, one from each of the two populations being compared.

IV. RESULTS

Comparison of zone of inhibition within the control and experiment groups (intra group comparison)

The zone of inhibition within each group was compared using repeated measures ANOVA

Group 1 (Control Group): The mean and standard deviation (Mean± S.D) for Porphyromonas gingivalis in Group I was 10.70 and 2.214 respectively with statistically highly significant p<.001. The mean and standard deviation (Mean± S.D) for Fusobacterium nucleatum in Group I was 4.40 and 2.011 respectively with statistically highly significant p<.001.

Group 2 (Experimental Group): The mean and standard deviation (Mean± S.D) for Porphyromonas gingivalis in Group II was 23.50 and 1.080 respectively with statistically highly significant p<.001. The mean and standard deviation (Mean± S.D) for Fusobacterium nucleatum in Group II was 10.60 and 0.966 respectively with statistically highly significant p<.001.

Group 3 (Experimental Group): The mean and standard deviation (Mean± S.D) for Porphyromonas gingivalis in Group III was 25.90 and 1.101 respectively with statistically highly significant p<.001. The mean and standard deviation (Mean± S.D) for Fusobacterium nucleatum in Group III was 14.40 and 1.174 respectively with statistically highly significant p<.001.

Group 4 (Experimental Group): The mean and standard deviation (Mean± S.D) for Porphyromonas gingivalis in Group IV was 32.20 and 1.033 respectively with statistically highly significant p<.001. The mean and standard deviation (Mean± S.D) for Fusobacterium nucleatum in Group IV was 19.00 and 1.155 respectively with statistically highly significant p<.001.

Comparison of Zone of inhibition between the Experimental groups (inter group comparison):

Group 1 (Control Group): On intergroup comparison of zone of inhibition of control group with other experimental groups for Porphyromonas gingivalis showed mean difference of -12.80, -15.20, -21.50 in group II, group III, group IV respectively and Fusobacterium nucleatum showed mean difference of -6.200*, -10.000*, -14.600* in group II, group III, group IV respectively with a statistically significant value p<.001.

Group 2 (Experimental Group): On intergroup comparison of zone of inhibition of Group II with other groups for Porphyromonas gingivalis showed mean difference of 12.80, -2.40, -8.70 in group I, group III, group IV respectively and Fusobacterium nucleatum showed mean difference of -6.200*, -3.800*, -8.400* in group I, group III, group IV respectively with a statistically significant value p<.001.

Group 3 (Experimental Group): On intergroup comparison of zone of inhibition of Group III with other groups for Porphyromonas gingivalis showed mean difference of 15.20, 2.40, -6.30 in group I, group II, group IV respectively and Fusobacterium nucleatum showed mean difference of 10.000*, 3.800*, -4.600* in group I, group II, group IV respectively with a statistically significant value p<.001.

Group 4 (Experimental Group): On intergroup comparison of zone of inhibition of Group IV with other groups for Porphyromonas gingivalis showed mean difference of 21.50, 8.70, 6.30 in group I, group II, group III respectively and Fusobacterium nucleatum showed mean difference of 14.600*, 8.400*, 4.600* in group I, group II, group III respectively with a statistically significant value p<.001.

Table 1: Inter-group comparison of inhibition zone for P gingivalis using ANOVA.

Group	N	Mean	Standard deviation	P value
Group I	10	10.70	2.214	<.001
Group II	10	23.50	1.080	
Group III	10	25.90	1.101	
Group IV	10	32.20	1.033	

Table 2: Tukey's post-hoc comparison of the above values

		Mean difference	P value
Group I	Group II	-12.80	<.001
	Group III	-15.20	<.001
	Group IV	-21.50	<.001
Group II	Group I	12.80	<.001
	Group III	-2.40	.004
	Group IV	-8.70	<.001
Group III	Group I	15.20	<.001

	Group II	2.40	.004
	Group IV	-6.30	<.001
Group IV	Group I	21.50	<.001
	Group II	8.70	<.001
	Group III	6.30	<.001

The zone of inhibition against Porphyromonas gingivalis was significantly greater for combination of PRF with Amoxicillin and Metronidazole, followed by PRF with Amoxicillin, PRF with Metronidazole, and PRF.

Table 3: Inter-group comparison of inhibition zone for Fusobacterium nucleatum using ANOVA.

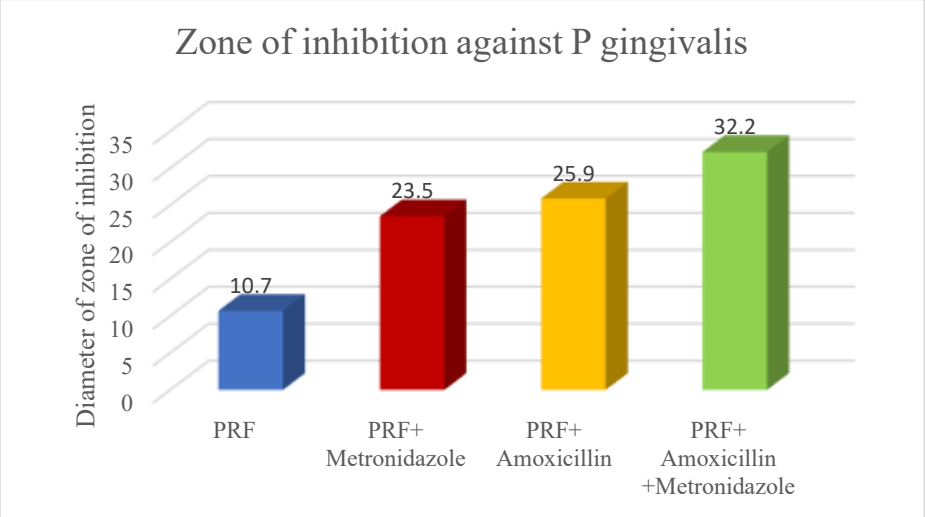
Group	N	Mean	Standard deviation	P value
Group I	10	4.40	2.011	<.001
Group II	10	10.60	.966	
Group III	10	14.40	1.174	
Group IV	10	19.00	1.155	

Table 4: Tukey's post-hoc comparison of the above values.

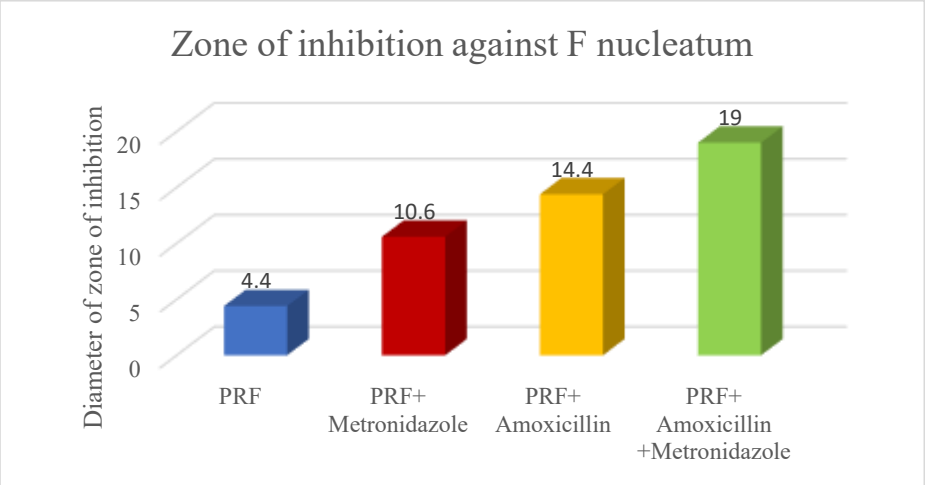
		Mean difference	P value
Group I	Group II	-6.200*	<.001
	Group III	-10.000*	<.001
	Group IV	-14.600*	<.001
Group II	Group I	6.200*	<.001
	Group III	-3.800*	<.001
	Group IV	-8.400*	<.001
Group III	Group I	10.000*	<.001
	Group II	3.800*	<.001
	Group IV	-4.600*	<.001
Group IV	Group I	14.600*	<.001
	Group II	8.400*	<.001
	Group III	4.600*	<.001

The zone of inhibition against Fusobacterium nucleatum was significantly greater for combination of PRF with Amoxicillin and Metronidazole, followed by PRF with Amoxicillin, PRF with Metronidazole, and PRF.

Graph 1: Inter-group comparison of inhibition zone for Porphyromonas gingivalis.



Graph 2: Inter-group comparison of inhibition zone for Fusobacterium nucleatum.



V. DISCUSSION

Wound healing after surgery always carries a risk of infection. Even with strict disinfection, bacteria can enter and colonize the wound, leading to tissue damage and delayed healing. Therefore, controlling infection is essential for a successful surgical outcome [15]. Although some evidence suggests benefits of peri- and post-operative systemic antibiotics in dental surgery, their side effects and the risk of antibiotic resistance make their routine use debatable [16].

The regenerative properties of platelet concentrates have been extensively studied over the past 20 years. However, their antimicrobial effects have been reported only in a few studies. The exact components responsible for this activity remain unclear, as platelet concentrates are a complex mix of platelets, white blood cells, and plasma. The specific roles of plasma and cellular components have not been thoroughly investigated. Current evidence indicates that platelets may contribute to antimicrobial defense by producing oxygen metabolites such as superoxide, hydrogen peroxide, and hydroxyl free radicals [17,18]. Additionally, platelets can bind to, aggregate, and internalize microorganisms, helping to remove pathogens from the bloodstream. They also participate in antibody-dependent cell cytotoxicity to target protozoal pathogens and release a variety of potent antimicrobial peptides [19,20]. Several factors have been suggested to contribute to their antimicrobial activity, including platelet-derived antimicrobial proteins, innate immune peptides, and components of platelet α-granules such as complement and complement-binding proteins [21,22].

Yeaman et al. (1997) proposed that the antimicrobial activity of platelet concentrates may result from the direct interaction of platelets with microorganisms, participation in antibody-dependent cell cytotoxicity, and the involvement of white blood cells in bacterial killing. Other suggested mechanisms include the release of myeloperoxidase, activation of antioxidant response elements, and antigen-specific immune responses. Activated platelets can also release growth factors that aid in ulcer healing and secrete platelet microbicidal proteins (PMPs) [18]. PMPs contain various antibacterial components, such as platelet factor 4, RANTES (regulated upon activation of normal T-cell expressed and secreted protein), connective tissue-activating peptide 3, platelet basic protein, thymosin beta-4, fibrinopeptide A, and fibrinopeptide B. These PMPs may act by interacting with bacterial membranes, altering membrane permeability, entering bacterial cells, and inhibiting the synthesis of macromolecules [20].

The aim of this study was to evaluate whether PRF incorporated with antibiotics could act as an antibacterial agent during the initial days of healing. Incorporating antibiotics into PRF showed a significant inhibition of anaerobic bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) compared to PRF alone. Using antibiotics in local delivery systems can provide high concentrations at the target site, potentially exceeding the minimum inhibitory concentration by over 1,000-fold [23,24].

Such high concentrations of antibiotics may negatively affect wound healing and exert cytotoxic effects on various cells. Certain antibiotics, like aminoglycosides, can be toxic to specific organs, including the kidneys and ears

[25]. To ensure practical relevance, this study used common antibiotic solutions at concentrations typically employed for intravenous administration in hospitals and clinics. **David Polak et al. (2019)**, in their in vitro study, reported that adding 0.5 ml of the tested antibiotic solutions to blood did not affect PRF formation and provided maximum antibacterial activity, while higher volumes were found to compromise PRF integrity [26].

Porphyromonas gingivalis and *Fusobacterium nucleatum* are well-known periodontal pathogens capable of invading human gingival epithelial cells and surviving within host tissues [28]. Both bacteria can persist in surrounding cells or tissues, causing infection and hindering wound healing [10]. For these reasons, they were chosen as the target microorganisms for the present study. Previous studies have shown that platelet-rich plasma exhibits antimicrobial activity against *Staphylococcus* aureus [9,10], as well as other microbes such as *Escherichia coli* [13], *Klebsiella pneumoniae* [11], *Enterococcus faecalis*, *Candida albicans*, and *Streptococcus oralis* [27]. However, the natural antibacterial effect of this blood product is considerably weaker than that of antibiotics [29]. Platelet-rich fibrin incorporated with all tested antibiotics showed significant inhibition of *Fusobacterium nucleatum* growth at all time points up to 96 hours post-preparation. PRF combined with clindamycin or penicillin also effectively inhibited the growth of *Staphylococcus aureus*. These findings indicate that antibiotics incorporated into PRF retain their activity for at least four days, supporting its potential use as a post-surgical slow-release antibacterial agent. Moreover, compressing PRF into a membrane-like form did not reduce its antimicrobial activity compared to the raw clot, suggesting that clinicians can use PRF in either form after adding antibiotics during preparation [26].

Additionally, modified forms of PRF, such as A-PRF, have demonstrated superior properties compared to standard L-PRF, including prolonged release of proteins and growth factors, as well as enhanced fibroblast migration, proliferation, and growth factor expression [30]. Combining antibiotics with A-PRF may further enhance its wound-healing benefits. This concept aligns with the idea proposed by **Miron and Zhang (2018)** [14], who discussed incorporating bioactive materials into liquid PRF to create an advanced local delivery system.

Therefore, this study aimed to enhance the antibacterial activity of PRF by incorporating different antibiotics and developing a protocol to prepare an antibiotic-releasing biomaterial (ARB) based on PRF. This approach could help prevent infections following minor surgical procedures. Local ARB may be particularly effective in situations where infection risk is high, potentially reducing the need for systemic antibiotics and minimizing associated side effects such as cytotoxicity and antibiotic resistance [31].

Several studies have attempted to enhance PRF. One study incorporated silver nanoparticles (SNP) to create SNP-modified PRF, which demonstrated improved mechanical properties and increased antimicrobial activity [32]. Another study evaluated the release kinetics of different antibiotics from PRF, showing that PRF could release antibiotics for up to a week. In the present study, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were selected due to their well-established roles as periodontal pathogens [31].

In the present study, antibacterial efficacy of Porphyromonas gingivalis and Fusobacterium nucleatum was significantly greater for combination of PRF with Amoxicillin and Metronidazole, followed by PRF with Amoxicillin, PRF with Metronidazole, and PRF alone. Based on the results of this study, in addition to the inherent benefits of PRF in wound healing, epithelialization, and growth factor release, PRF combined with antibiotics can serve as an effective vehicle for local drug delivery.

The concept of an antibiotic-releasing biomaterial (ARB) introduced in this study may have significant clinical relevance, as surgical site infections remain a common complication following minor procedures. ARB could play a key role in reducing infections that interfere with secondary healing after periodontal treatment, by providing gradual local antibiotic release. Further research is recommended to test this protocol with different antibiotics and target bacteria to advance this concept toward clinical application.

VI. CONCLUSION

In the present study, Platelet- rich fibrin clots were formed with the incorporation of 0.5ml of antibiotics (Metronidazole 5 mg/ml, Amoxicillin 50mg/ml, combination of Metronidazole 5mg/ml and Amoxicillin 50mg/ml) and its anti-bacterial efficacy was examined using anti-biogram assay on standard strains of *Porphyromonas gingivalis* and *Fusobacterium nucleatum.*

The results of the present study shows that the antibacterial efficacy of Porphyromonas gingivalis and Fusobacterium nucleatum was significantly greater for combination of PRF with Amoxicillin and Metronidazole, followed by PRF with Amoxicillin, PRF with Metronidazole, and PRF alone.

This study offers clinicians a novel approach to controlling post-operative infections using PRF modified with antibacterial properties. Such a material could serve as a valuable topical surgical tool, promoting tissue healing while preventing local infection. Additionally, its use may reduce the reliance on systemic antibiotics. However, the clinical application of these in vitro findings should be approached with caution. The antibacterial effectiveness of the modified PRF, as well as any potential impact on the natural healing properties of L-PRF, should be validated in animal models and clinical studies.

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