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Antimicrobial Effects of Platelet-rich Plasma against Selected Oral and Periodontal Pathogens

REHAM L. AGGOUR[1]* and LINA GAMIL[2]

[1] Oral Medicine and Periodontology Department, Faculty of Dentistry, October 6th University, 6th October City, Giza Governorate, Egypt
[2] Microbiology Department, Faculty of Pharmacy, October 6th University, 6th October City, Giza Governorate, Egypt

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Abstract

Antimicrobial properties of platelet rich plasma (PRP) against various microorganisms have been recently pointed out. PRP could be an alternative to conventional antibiotics in preventing oral and periodontal infections. We examined whether PRP has *in vitro* antimicrobial properties against *Aggregatibacter* actinomycetemcomitans, *Prophyromonas gingivalis*, *Staphylococcus aureus* and *Candida albicans*. PRP and platelet-poor plasma (PPP) were obtained from whole blood of 10 healthy volunteers and 10 periodontitis patients. *In vitro* laboratory susceptibility was carried out using the modified agar diffusion method by measuring the diameters of inhibition zones on agar plates coated with selected microbial strains. Both calcium chloride (CaCl2) activated and non activated samples were tested. Both activated PRP and PPP, of both patients and controls, effectively inhibited the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans*. However, a statistically significant difference in favor of PRP was found indicating more susceptibility to PRP than PPP ($p < 0.05$). Non activated PRP and PPP exhibited negative zones of inhibition against the studied microorganisms. There was no activity against *S. aureus*. No statistically significant difference was found between the antimicrobial effects of PRP and/or PPP obtained from patients and controls ($p > 0.05$). We conclude that PRP is a potentially useful substance against oral and periodontal pathogens. Activated PRP was found to be more active than activated PPP and the activation of coagulation is a fundamental step. Additionally, the antimicrobial activity of PRP and/or PPP seems not to be affected by periodontitis.

Key words: antimicrobial activity, oral infection, platelet-rich plasma

Introduction

Platelet-rich-plasma (PRP) is an autologous concentration of platelets in plasma developed by gradient density centrifugation. It has been increasingly used in a variety of medical fields. The effectiveness of this procedure lies in the delivery, up on stimulation, of a wide range of platelet growth factors mimicking the physiologic wound healing and reparative tissue processes (Werner and Grose, 2003). Current evidence suggests that platelets have multiple functional attributes indicative of an integral role in antimicrobial host defense (Yeaman *et al*., 1998; Tang *et al*., 2002). These functions include navigation toward the inflammatory chemoattractant N-f-etLeuPhe, expression of Fc and complement C3a/C5a receptors, and the capacity to generate antimicrobial oxygen metabolites. Platelets interact directly with microorganisms, contribute to clearance of pathogens from the bloodstream, and significantly participate in antibody dependent cell cytotoxicity against microbial pathogens (Yeaman, 1997; Yeaman and Bayer, 1999). Moreover, platelet α-granules contain catecholamines, serotonin, osteonectin, von Willebrand factor, proaccelerin and other substances (Anitua *et al*., 2005; Bielecki *et al*., 2006). These are released in high concentrations after platelet aggregation and may also have antibacterial effects. Yeaman *et al*. (1998) and Tang *et al*. (2002) had shown that platelet microbicidal proteins released after platelet activation demonstrate potent activities against many gram-negative, gram-positive and fungal pathogens. Collectively, these findings suggest that human platelets possess, and can be stimulated to release, several antimicrobial polypeptides (Krijgsveld *et al*., 2000). The antimicrobial potential of

* Corresponding author: R.L. Aggour, Oral Medicine, Periodontology Oral Diagnosis and Oral Radiology, Faculty of Dentistry, October 6 University, 6th October City, Giza Governorate, Egypt; **e-mail: rehamaggour@rocketmail.com**

platelets might be increased through their concentration in PRP (Drago *et al*., 2013).

Despite extensive use in clinical procedures, only few studies have investigated PRP's microbicidal activity (Bielecki *et al*., 2007; Moojen *et al*., 2008; Anitua *et al*., 2012; Burnouf *et al*., 2013; Drago *et al*., 2013). Mariani *et al.* (2015) tested the effect of PRP against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*, as species potentially involved in bone, soft tissue and wound infections (Zalavras *et al*., 2004). In the current study, we have selected four microorganisms that are commonly found in oral and periodontal infections and investigated whether PRP could have *in vitro* antimicrobial effects against them.

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are major periodontal pathogens. *A. actinomycetemcomitans* is a nonmotile, capnophilic, Gram-negative, coccobacillus, and a facultative anaerobe. *P. gingivalis* is a nonmotile, coccobacillus, and an obligate anaerobe. Both pathogens produce a number of virulence factors and damaging metabolites that are associated with initiation and progression of periodontal destruction. Adjunctive antimicrobial therapy along with appropriate mechanical therapy helped in the elimination of these pathogens. The linking of the inductive and antimicrobial effects of PRP may be beneficial for the treatment of periodontal disease (Teles *et al*., 2006).

S. aureus has been associated with dentoalveolar infections and oral mucosal lesions. Studies suggested that the presence of *S. aureus* in saliva was a significant risk factor for aspiration pneumonia (Terpenning *et al*., 2001; El-Solh *et al*., 2004). Oral *S. aureus* may also serve as a reservoir for cross-infection to other patients, as well as health care staff (Smith *et al*., 2003). Additionally, some studies have demonstrated that *S. aureus* plays a role in exacerbating dental diseases by forming a biofilm with the causative pathogens of periodontal diseases (Smith *et al*., 2001; Cuesta *et al*., 2010; Passariello *et al*., 2012).

Oral candidiasis is the most common fungal infection encountered in general dental practice. It manifests in a variety of clinical presentations and can occasionally be refractory to treatment. It is caused by commensal *Candida* species. The most commonly implicated strain is *C. albicans* which is isolated in over 80% of oral candidal lesions (Farah *et al*., 2010).

The development of new, cost-effective and safe measures to prevent and control oral and periodontal infections is important. The present study was undertaken to test the *in vitro* antimicrobial activity of activated and non activated PRP and PPP against *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans*.

Experimental

Materials and Methods

Study design and donors. The study was conducted in compliance with good clinical practice guidelines and the Declaration of Helsinki. The subjects of this study were patients who visited the outpatient clinic of the Faculty of Dentistry, October 6 University. Ten patients diagnosed with generalized chronic periodontitis (Armitage, 1999) (mean age ± SD: 45.2 ± 3.1 years) and ten periodontally healthy subjects with no clinical attachment loss and a probing pocket depth less than or equal to 3mm (mean age ± SD: 42.7 ± 3.4 years) were included. All subjects received verbal and written information about the study and signed consent forms before enrollment in the study. Criteria for exclusion were the use of antibiotics or other antimicrobial and/or anti-inflammatory medicaments during the previous 3 months, smoking, pregnancy, suffering from systemic disorders and/or presence of infection. Subjects with hemoglobin concentrations < 11 g/dl and platelet numbers $\leq 150 \times 10^3$/µl, were also excluded from the study.

Collection of samples. Sub-gingival samples were taken from the ten chronic periodontitis patients after rinsing the mouth with distilled water. Samples from the sub-gingival pockets were collected with a Gracey 7/8 curette, after relatively isolating the zone with cotton rolls and high-power suction and removing supra gingival biofilm. Each patient contributed a total of four samples collected from the deepest pocket of each patient's dentition quadrant. Samples from all patients (40 samples) were placed in sterile PBS (phosphate buffer solution, pH 7.4) transport medium, and kept at –4°C until processing. The samples were processed within 6 hours.

Microbiological procedures. The isolates were all purified and identified to species level. Tenfold serial dilutions were prepared in sterile phosphate buffered saline. Appropriate dilutions were plated onto non-selective 5% horse blood agar plates (Oxoid no. 2; Oxoid Ltd, Basingstoke, England) supplemented with haemin (5 Fg/l) and menadione (1 Fg/l) (BA), and on trypticase soy-serum bacitracin-vancomycin (TSBV) plates (Slots, 1982) for selective isolation and growth of *A. actinomycetemcomitans.* TSBV plates were incubated at 35°C in 10% CO_2/90% N_2 for 4 days.

Identification of isolates. Microscope studies with Gram coloring and biochemical tests were used to identify the microorganisms. Identification of *A. actinomycetemcomitans* and *P. gingivalis* isolates was carried out using the criteria and techniques of Slots (1986; 1987). *P. gingivalis* was identified based on microscopic morphology, Gramreaction and detection of a trypsin-like activity based on the degradation of benzoyl-DL-

arginine-2-naphthylamide (Sigma, St Louis, MO, USA) (van Winkelhoff *et al.*, 1988). Identification of *A. actinomycetemcomitans* was based on the characteristic colony morphology (star-like inner structure) on the TSBV plate, and a positive catalase reaction upon exposure to 3% H_2O_2. *S. aureus* were grown in plates containing Mannitol Salt Agar (Becton Dickinson, USA) and was identified in this media by the production of golden color zones around the colonies. Additionally, catalase and coagulase tests were performed (Murray *et al.*, 1999).

For the isolation and identification of *C. albicans*, we used the exhaustion technique in plates of chromogenic agar media, CandiSelect (Bio-Rad, France), which is a specific culture media for the growth of yeasts. Blue colonies were identified as *C. albicans*.

Platelet rich plasma preparation. PRP was prepared following a one-step procedure according to Anitua's protocol (Anitua *et al.*, 2008). Peripheral blood (4.5 ml) from each donor was taken by venipuncture into 5 ml blood-collecting tubes containing 0.5 ml of sodium citrate solution (3.8%) as anticoagulan and centrifuged using a laboratory centrifuge (Centurion, Scientific limited, UK) at 460 × g for 8 min, thus obtaining three layers: platelet-poor plasma (PPP) on the top of the tube, PRP in the middle and erythrocytes at the bottom of the tubes. Subsequently, PPP component was removed. PRP, located on the red blood cell pellet, was carefully harvested avoiding leukocyte collection. The platelet and leukocyte counts in the peripheral blood, PRP and PPP were measured automatically using a hematology analyzer. According to Anitua *et al.* (2008) platelet concentration in PRP is at least twice the concentration in whole blood; while leukocyte concentration is consistently lower ($< 10^3$ white blood cells/μl). Platelets should be almost absent in PPP. PRP and PPP were activated shortly before use by the addition of 10% calcium chloride (50 μl per ml of PRP or PPP).

Determination of antimicrobial activity. Antimicrobial activity of PRP, PPP against *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans* were carried out according to the modified agar diffusion method. Both CaCl2 activated and non activated samples were tested. Controls were performed using CaCl2 *per se* at a final concentration of 4.5 mM.

An overnight culture of *S. aureus* and *C. albicans* and three day culture of *A. actinomycetemcomitans* and *P. gingivalis* were adjusted to reach a cell density of approximately 10^7 to 10^8 cfu/ml and was surface inculcated on BHA plates, then fixed volumes of PRP with CaCl2, PPP with CaCl2 and CaCl2 were aseptically added, by using separate micropipettes, to cups made in these plates. Plates were incubated for 24 h for *S. aureus* and *C. albicans* at 37°C and for 3–7 days under microaerophilic condition for *A. actinomycetemcomitans* and *P. gingivalis*. Twenty four hours after incubation, activity was assessed by measuring the zones of inhibition, which were recorded as the diameter in millimetres across the centre of the embedded discs.

Fig. 1. Antimicrobial activity of platelet rich plasma against *A. actinomycetemcomitans* using disc diffusion method.

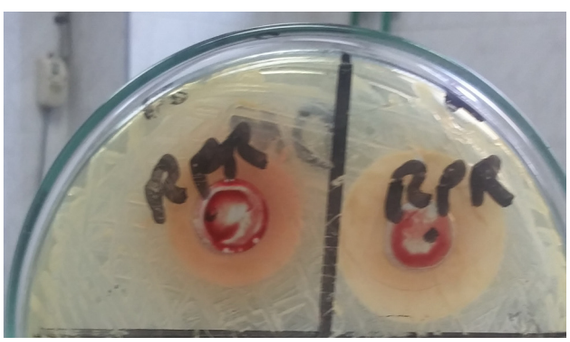

Fig. 2. Antimicrobial activity of platelet rich plasma against *P. gingivalis* using disc diffusion method.

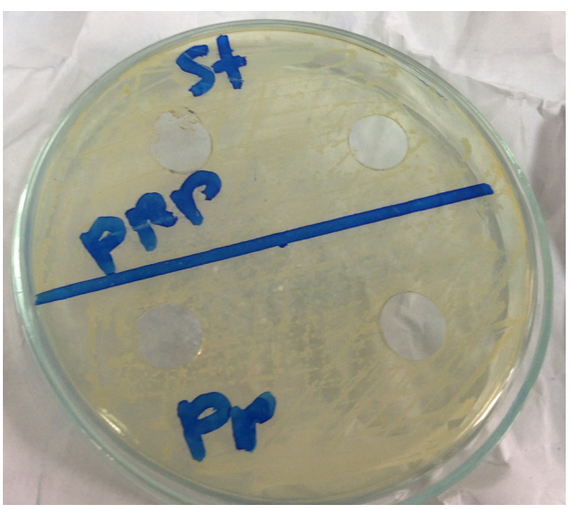

Fig. 3. Antimicrobial activity of platelet rich plasma and platelet poor plasma against *S. aureus* using disc diffusion method.

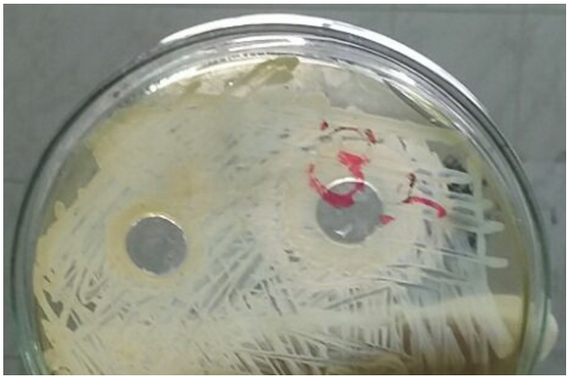

Fig. 4. Antimicrobial activity of platelet poor plasma and calcium chloride against *P. gingivalis* using disc diffusion method.

Statistical analysis. The data are presented as the mean ± SD. Statistical differences were evaluated using the Mann-Whitney U test with a p-value ≤ 0.05 considered to be significant.

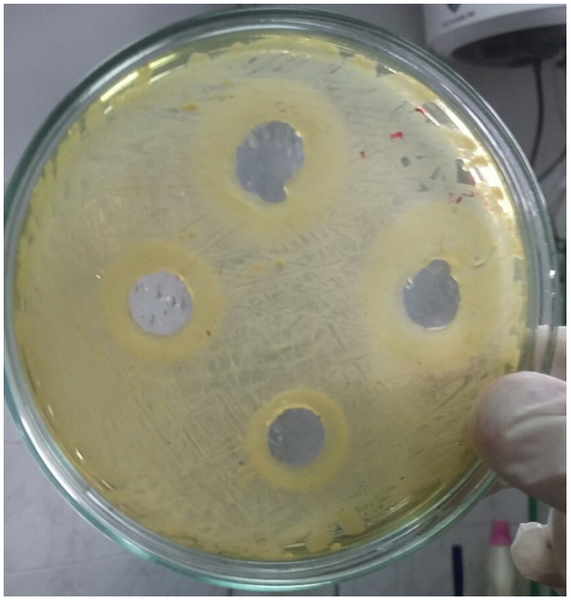

Fig. 5. Antimicrobial activity of platelet rich plasma, platelet poor plasma and calcium chloride against *C. albicans* using disc diffusion method.

Results

No significant difference has been found between platelet concentration in PRP pooled blood samples of periodontitis patients and controls ($p > 0.05$). It has been found to be about 2.5 times higher than in whole blood (Table I). Platelets were almost absent in PPP of both patients and controls ($< 10^2$ platelets/ml) (Table I). All pooled PRP and PPP was efficiently leukocyte-depleted ($< 10^3$ white blood cells/ml).

In vitro antimicrobial activity of PRP and PPP (patients and controls) showed that both of them effectively inhibited the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* but not *S. aureus*. A statistically significant difference in favor of PRP has been found indicating more susceptibility to PRP than PPP ($p < 0.05$). Only activated samples displayed an antimicrobial activity, whereas non activated samples did not exhibit any effect, suggesting that the activation of coagulation is a fundamental step (Table II, III).

Among the tested microorganisms *A. actinomycetemcomitans* was found to be the most sensitive to both PRP and PPP while *C. albicans* the least. Activated PRP of the 10 periodontitis patients exhibited mean zones of inhibitions of 29 ± 0.1, 25 ± 0.4 and 20 ± 0.1 mm against *A. actinomycetemcomitans, P. gingivalis* and *C. albicans* respectively. While activated PPP of the same patients exhibited mean zones of inhibition of 25 ± 0.4, 18 ± 0.3 and 17 mm against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* respectively (Table II). Activated PRP of 10 controls exhibited mean zones of inhibitions of 30 ± 0.2, 26 ± 0.6, 19 ± 0.05 mm against *A. actinomycetemcomitans, P. gingivalis* and *C. albicans* respectively and activated PPP exhibited mean zones of inhibitions 23 ± 1, 20 ± 1.1 and 15 ± 0.99 mm against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* respectively (Table III). Calcium chloride *per se* exhibited zones of inhibitions of 17 ± 03, 12 ± 0.1, 14 ± 0.3 mm against

Table I
Characteristics of the sample presented as mean ±SD

Variable	Periodontitis group (Mean ± SD)	Control group (Mean ± SD)	(p-value)
Age(years)	45.2 ± 3.1	42.7 ± 3.4	> 0.05
Gender Male Female	 **6** **4**	 **5** **5**	> 0.05
Mean platelet concentration in whole blood (platelets /ml)	215×10^3	210×10^3	> 0.05
Mean platelet concentration in PRP (platelets /ml)	480×10^3	462×10^3	> 0.05
Mean platelet concentration in PPP (platelets /ml)	–	–	> 0.05

Table II
Microbiological screening for the antimicrobial activity of the activated PRP, activated PPP, non-activated PRP and non-activated PPP obtained from 10 periodontitis patients and $Cacl_2$ *per se* against *A. actinomycetemcomitans*, *P. gingivalis*, *C. albicans* and *S. aureus* by agar diffusion method

Name of microorganism	Diameter of zone of inhibition ± standard deviation (SD) in mm					
	Activated PRP	Non-activated PRP	Activated PPP	Non-activated PPP	$Cacl_2$	PRP *vs* PPP (*p*-value)
A. actinomycetemcomitans	29 ± 0.1	–	25 ± 0.4	–	17 ± 03	< 0.05
P. gingivalis	25 ± 0.4	–	18 ± 0.3	–	12 ± 0.1	< 0.05
C. albicans	20 ± 0.1	–	17	–	14 ± 0.3	< 0.05
S. aureus	–	–	–	–	–	

– No inhibition zone

Table III
Microbiological screening for the antimicrobial activity of the activated PRP, activated PPP, non-activated PRP and non-activated PPP obtained from 10 controls and $Cacl_2$ *per se* against *A. actinomycetemcomitans*, *P. gingivalis*, *C. albicans* and *S. aureus* by agar diffusion method

Name of microorganism	Diameter of zone of inhibition ± standard deviation (SD) in mm					
	Activated PRP	Non-activated PRP	Activated PPP	Non-activated PPP	$Cacl_2$	PRP *vs* PPP (*p*-value)
A. actinomycetemcomitans	30 ± 0.2	–	23 ± 1	–	17 ± 03	< 0.05
P. gingivalis	26 ± 0.6	–	20 ± 1.1	–	12 ± 0.1	< 0.05
C. albicans	19 ± 0.05	–	15 ± 0.99	–	14 ± 0.3	< 0.05
S. aureus	–	–	–	–	–	

– No inhibition zone

A. actinomycetemcomitans, P. gingivalis and *C. albicans* respectively (Table II, III). Activated PRP and Activated PPP of patients and controls exhibited negative zone of inhibition against *S. aureus.*

The difference in the antimicrobial activity between PRP and PPP of patients and controls against the studied microorganisms was not significant ($p > 0.05$).

Discussion

It is impossible to separate wound healing and infection. The use of PRP may be advantageous compared to conventional antibiotic treatments since PRP is less likely to induce antibiotic resistance and PRP's antimicrobial and healing-promoting properties may have a synergistic effect on infection prevention. Moreover, PRP is inherently biocompatible, and safe. In the current study, we tested the antimicrobial activity of PRP, PPP, with and without activation against four microorganisms, *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans.* We used samples from both periodontally healthy individuals and patients with chronic periodontitis to investigate potential donor variations and whether the antimicrobial effect of PRP could be affected by the presence of chronic periodontitis.

Although leukocytes have been proposed as an additional source for cytokines, microbicidal proteins and myeloperoxidase activity (Hampton *et al*., 1996), the few available published data do not support the increased microbicidal activity of PRP due to the leukocyte component (Bielecki *et al*., 2007; Moojen *et al*., 2008; Anitua *et al*., 2012). Therefore in this study we used the Anitua's protocol (Anitua *et al*., 2008) that allows the production of leukocyte-poor PRP. Originally bovine thrombin was used as an activating agent, but the rare and major risk of coagulopathy from antibody formation has restricted the routine use of bovine thrombin. Calcium chloride, used in the current study, offer an alternative *in vitro* activation means (Anitua, 1999).

In the current study, we have found that PRP, and to a lesser extent, PPP has antimicrobial effects against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* but not *S. aureus*. Only activated samples were able to inhibit microbial growth, suggesting that the activation of coagulation is a fundamental step. No significant difference was found between *in vitro* antimicrobial effect of PRP and PPP isolated from periodontitis patients and healthy controls.

To the best of our knowledge, only Yang *et al.* (2015) have investigated the antimicrobial activity of PRP and other plasma preparations against periodontal

pathogens. The authors found that all plasma preparations can inhibit bacterial growth, with PRP showing the superior activity. In the current study, the activity of PRP against *A. actinomycetemcomitans* and *P. gingivalis* is comparable to that of Yang *et al.* (2015). These results confirm the beneficial role of PRP in the treatment of periodontal disease.

Activated PRP and activated PPP didn't inhibit the growth of *S. aureus*. These results are contradictory to those obtained by Bielecki *et al.* (2007). They performed a microbiological examination of PRP on 20 healthy volunteers; a strong activity comparable to gentamicin and oxacillin for PRP against methicillin-sensitive *S. aureus* was demonstrated. According to the former authors, PRP also inhibited the growth of methicillin – resistant *S. aureus* and *Escherichia coli*. Moreover, a study by Moojen *et al.* (2008) investigated the effect of various platelet concentrate on *S. aureus*, they found a strong effect of PRP activated by thrombin (PLG) but not non activated PRP. They concluded that the decrease in microbial load is mainly attributed to the effect of activation by thrombin. However, they found that the strong antimicrobial effect of PLG seems to be limited to the first hours after application. Additionally, Jia *et al.* (2010) reported that PRP forms into PLG after activation with bovine thrombin can inhibit *S. aureus* reproduction *in vitro* and can effectively prevent bone infection *in vivo*. The reasons for the inconsistency between their results and the results of the current study may be explained by differences in the bacterial isolates and the increased frequency of antimicrobial use in minor surgical procedures which may lead to increased antimicrobial resistance in our population. Additionally, in the current study, CaCl2, which may have a weaker and more prolonged activation time, has been used for activation instead of thrombin (Li *et al.*, 2013). Moreover, the different protocol used for PRP preparation can lead to products with different cellular components and biological characteristics. The difference in the sensibility of the method (modified agar diffusion method) used to evaluate the susceptibility to platelet concentrate should also be considered (Ehrenfest *et al.*, 2013; Assirelli *et al.*, 2014; Cavallo *et al.*, 2014).

Another important obtained result is the observation that PRP and PPP were less active against *C. albicans* than against *A. actinomycetemcomitans* and *P. gingivalis*. This result is consistent with the findings of Tang *et al.* (2002), who tested *in vitro* antimicrobial activity of antimicrobial peptides isolated from human platelets, and noticed that they were more potent against bacteria than fungi.

In contrary to Li *et al.* (2013) study, PPP has demonstrated antimicrobial effect. However, a greater susceptibility for activated PRP was observed. The antimicrobial effect of PPP might be explained by the presence of antimicrobial peptides and other factors in plasma, either released from the platelets during normal functioning or by breakdown of some platelets during the blood processing (Wu *et al.*, 1994).

Future research should focus on the analysis of the contribution of individual PRP and PPP components, its antimicrobial capacity compared to antibiotics and its exact antibacterial spectrum. Moreover, despite the clinical relevance of these results, it is clear that the *in vitro* behavior of bacterial plates may not mimic the *in vivo* environment of the oral cavity and *in vitro* susceptibility to antimicrobials should only be regarded as a guide to potential *in vivo* activities. Future clinical trials should be done to investigate the potential practical implications of the findings of this study as well as the modality of clinical application of platelet concentrates.

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