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IN VITRO RESEARCH

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Incorporating antibiotics into platelet-rich fibrin: A novel antibiotics slow-release biological device

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Abstract

Background: The aim of the present in vitro study was to explore the possibility of using platelet-rich fibrin (PRF) as a local sustained released device for antibiotics.

Materials and methods: Platelet-rich fibrin was prepared with the addition of antibiotics (5 mg/ml metronidazole; 150 mg/ml clindamycin; 1 mU/ml penicillin) or saline prior to centrifugation, while collagen sponges served as control. PRFs anti-bacterial properties were examined in an anti-biogram assay with *Staphylococcus aureus* or *Fusobacterium nucleatum* at different time intervals after PRF preparation.

Results: The addition of antibiotic solutions at volumes of 2 or 1 ml led to significant changes in PRF's physical properties, while the addition of 0.5 ml solution did not. PRF with saline showed minor anti-bacterial activity, while all PRFs with antibiotics showed significant anti-bacterial activity ($p < 0.05$). No differences were observed between raw (clot) and pressed (membrane) forms of PRF. Collagen sponges with and without antibiotics showed similar results to PRF. PRF and collagen sponges with antibiotics preserved their anti-bacterial properties 4 days after preparation.

Conclusions: Platelet-rich fibrin incorporated with antibiotics showed long-term anti-bacterial effect against *F. nucleatum* and *S. aureus*. This modified PRF preparation may be used to reduce the risk of post-operative infection in addition to the beneficial healing properties of PRF.

1 | INTRODUCTION

Platelet concentrates are obtained after processing whole blood through centrifugation. These concentrates include leucocyte- and platelet-rich plasma (L-PRP) and pure platelet-rich plasma (P-PRP) or rich in growth factors (PRGF)) (Dohan Ehrenfest et al., 2014). However, difficulties in preparation and inconsistent outcome led to the development of leucocyte- and platelet-rich fibrin (L-PRF, herein PRF). Unlike L-PRP and P-PRP, the preparation and the use of PRF are simple and do not require addition of anticoagulants (Castro et al., 2017).

Following obtaining autogenous blood via venipuncture, the centrifuged blood results in three layers: red blood corpuscles (RBC), platelet-poor plasma (PPP) and an intermediate layer of PRF. The PRF layer is rich in fibrin, platelets, leucocytes, monocytes and growth factors (Castro et al., 2017). The use of PRF was found to promote microvascularization, migration of epithelial cell and accelerated healing (Dohan et al., 2006). Its use has been reported for many surgical applications: treatment of bony defects, dental implant surgery, post-extraction healing and reducing rate of post-surgery complications (Canellas, Ritto, & Medeiros, 2017; Ozgul et al., 2015). The PRF can be used in its raw (clot) form or compressed to achieve a membrane-like form.

Wound healing following surgery always bares a risk of infection (Yang et al., 2015). Even when disinfection has been stringently enforced, microbes can infiltrate and colonize the underlying wound tissues, resulting in loss of tissue integrity in the surgical site and impaired healing (Yang et al., 2015). Therefore, infection control is a prerequisite for successful surgical procedure (Yang et al., 2015). Weak evidence exists for the beneficial use of peri- and post-operative

systemic antibiotics following dental surgery (Kreutzer, Storck, & Weitz, 2014), and their adverse effects with the possibility for the development of resistant bacteria make their use controversial (Lodi et al., 2012).

Cieslik-Bielecka, Gazdzik, Bielecki, and Cieslik (2007) showed that platelet-rich gels lacks anti-bacterial properties while producing the gel 30 min after intravenous administration of amoxicillin and clavulanic acid (Augmentin) rendered the gel with strong anti-bacterial activity against *Enterococcus faecalis* (Cieslik-Bielecka et al., 2007). Furthermore, Miron and Zhang, in an extensive review, discussed the possibility of combining various bioactive materials with liquid PRF to produce an advanced local delivery system for small and large biomolecules (Miron & Zhang, 2018). Taken together, instead of systemic antibiotic administration to patients prior to PRF preparation, as suggested by Cieslik-Bielecka et al., an approach to combine antibiotics to the PRF not via its systemic administration would be ideal. Still, to date, no method provided evidence of the ability to incorporate antimicrobial agent into PRF.

The aim of the study was to establish a simple and practical method that gives antimicrobial properties to PRF and to provide in vitro evidence of its effectiveness. Such product may provide additional advantage to known beneficial healing properties of PRF and reduces the need for systemic antibiotics in a variety of oral surgical procedures.

Clinical Relevance

Scientific rational for the study: Autogenous platelet-rich fibrin (PRF) promotes healing following oral surgery. The current study aimed to further improve PRF by providing it with anti-bacterial activity.

Principal findings: Addition of metronidazole, penicillin or clindamycin to PRF produced antimicrobial preparation with long-term anti-bacterial activity, up to 4 days of the experiments.

Practical implications: Using PRF with antibiotics may reduce the risk of post-operative infection and augment the beneficial healing properties of PRF.

2 | MATERIALS AND METHODS

2.1 | PRF preparation

Platelet-rich fibrin preparation was carried out according to Fujioka-Kobayashi et al. (2017). In brief, blood samples were collected from 5 healthy volunteers into 10-ml collection glass tubes without anti-coagulants by a venous puncture (PRF Process, Nice, France). The tubes were immediately centrifuged at 2,700 rpm (RCF-max of 735 g (Miron, Choukroun, & Ghanaati, 2018)) for 12 min at room temperature using a fixed angle centrifuge (PRF Process, Nice, France). The fibrin clot located in the middle fraction of the centrifuged blood was collected using sterile tweezers and separated from the RBC base using sterile scissors.

2.2 | Antibiotics incorporation into PRF

Prior to tubes' centrifugation, antibiotics were added to the fresh blood using a syringe at volumes of 0.5, 1 and 2 ml. The following antibiotics and concentrations were tested: metronidazole 5 mg/ml, penicillin 1 mU/ml or clindamycin 150 mg/ml. Saline (in similar volumes) was used as control. Experiments were carried out with PRF in its raw (clot) form or following compression (membrane).

2.3 | PRF physical properties measurement

The following physical characteristics were recorded: consistency, length and total volume. Consistency was described as a non-parametric narrative. Length was measured using a University of North Carolina (UNC) probe (Hu-Friedy, Chicago, IL, USA). Volume measurement was carried out with the PRF clot: each PRF was inserted into a measurement tube with 10 ml of saline. Changes in volume inside the tube were recorded with an accuracy of 0.1 ml.

2.4 | Collagen sponge preparation

Collagen wound dressing material was used as control for the PRF (Collatape, Zimmer Biomet, Carlsbad, USA). Double distilled water (10 ml) was placed in collection tubes (similar to preparation of PRF) with or without the addition of antibiotics (0.5 ml) and the collagen dressing. Following incubation at room temperature for 12 min (similar to the PRF preparation time), the collagen dressings were collected for analysis.

2.5 | Anti-biogram assay

Staphylococcus Aureus (clinical isolate) was cultured in brain heart infusion broth (Becton Dickinson, Sparks, USA) in aerobic condition at 37°C for 24 hr. *Fusobacterium nucleatum* (PK1594) was cultured in Wilkins broth (Oxoid, Basingstoke, UK) in anaerobic condition at 37°C for 24 hr. Both bacteria were washed separately in phosphate-buffered saline (PBS) and seeded evenly (grass seeding) on blood agar plates (Hy laboratories, Rehovot, Israel). The anti-biogram assay was performed immediately after PRFs and collagen sponges' preparation. The tested material (PRF clot, PRF membrane or collagen sponge) was placed at the middle of the plate. The plates were transferred to aerobic or anaerobic conditions for *S. aureus* and *F. nucleatum*, respectively, at 37°C for 7 days. The anti-bacterial activity (susceptibility) of the tested materials was determined by measuring the diameters of CFUs free area. Diameter was measured twice per sample (with one measurement perpendicular to the other) using a UNC probe, and the mean of both measurements was used as the inhibition value of the sample. Results are expressed as the mean of all diameter measurements of the samples.

TABLE 1 Changes of platelet-rich fibrin (PRF') physical characteristics following addition of antibiotics

	Added volume (ml)	PRF length (mm)	Compression properties	Separation from RBC base
Control		15	Remained stable	Separated with scissors
Metronidazole	0.5	15	Remained stable	Almost separated
	1	2	Too small to compress	Small piece floating in yellow phase
	2	10	Lost during compression	Almost separated
Penicillin	0.5	17	Remained stable	Spontaneous separation
	1	25	Lost during compression	Almost separated
	2	25	Remained stable	Almost separated
Clindamycin	0.5	20	Remained stable	Almost separated
	1	20	Lost during compression	Spontaneous separation
	2	13	Lost during compression	Spontaneous separation

Note. Physical properties of PRF at its clot form alone (control) or following incorporating metronidazole/penicillin/clindamycin at volumes of 0.5, 1 and 2 ml.

Time-depended anti-bacterial activity was tested using PRFs and collagen sponges that were kept in phosphate-buffered saline (10 ml) at 37°C for 24, 48, 72 and 96 hr—and then used in the anti-biogram assay as described above.

2.6 | Statistical analysis

All experiments were performed in triplicates and repeated at least three times. The data were analysed using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated measurements analysis of variance (RM ANOVA) was applied to test the significance of the differences between the treated groups. If the differences were found to be significant, inter-group differences were tested for significance using Student's *t* test with Bonferroni correction for multiple testing.

3 | RESULTS

3.1 | Correlation of PRF physical properties with the addition of different volumes of antibiotics solutions

Changes in the physical properties in PRFs (fragility, uncompressible or too delicate for manipulation) with volumes > 0.5 ml rendered them inadequate for further experiments (Table 1). The addition of 0.5 ml of all tested solutions did not change the physical properties of the PRF and was therefore set as the following standard antibiotics volume that was added to the PRF in the following experiments (Table 1).

Quantification of PRFs' volumes showed small, but statistically significant changes between control PRF and PRF with the addition of 0.5 ml solution. Interestingly, the volume of PRF + antibiotics groups was higher than the PRF alone (Figure 1, $p < 0.05$).

3.2 | Anti-bacterial effect of PRF with antibiotics vs. natural PRF

In a preliminary anti-bacterial susceptibility assay (measuring the ability of an antibiotics to completely inhibit bacterial growth in culture), *F. nucleatum* was found to be susceptible to all tested antibiotics (penicillin, clindamycin and metronidazole). *S. aureus* was found to be susceptible to penicillin and clindamycin, but partially resistant to metronidazole (data not shown).

Since the added antibiotics may be trapped in the liquid phase of the PRF or within the PRF protein structure, we tested PRF in its clot form as well as its pressed (membrane) form. The membrane form of PRF contains only small volume of fluid, thus providing evidence of entrapment of the antibiotics within the PRF.

PRF with saline showed little anti-bacterial activity in its clot and membrane forms against the two tested microorganisms (Figure 2), while all PRFs with antibiotics showed significant anti-bacterial activity ($p < 0.05$). The anti-bacterial effect against *S. aureus* was significantly higher with clindamycin and penicillin compared with metronidazole (Figure 2, $p < 0.05$), while the anti-*F. nucleatum* activity was higher with clindamycin and metronidazole compared with penicillin ($p < 0.01$). No significant differences were observed between the clot and pressed forms of the PRF.

We used commercially available collagen sponges as a control carrier for the antibiotics. Collagen sponge alone did not show any anti-bacterial activity (Figure 2). The addition of all tested antibiotics rendered the sponge with anti-*S. aureus* activity similar to the PRF results. Clindamycin or metronidazole within the sponge showed anti-*F. nucleatum* activity at lower levels than the PRF carrying the same antibiotics (Figure 2, $p < 0.05$).

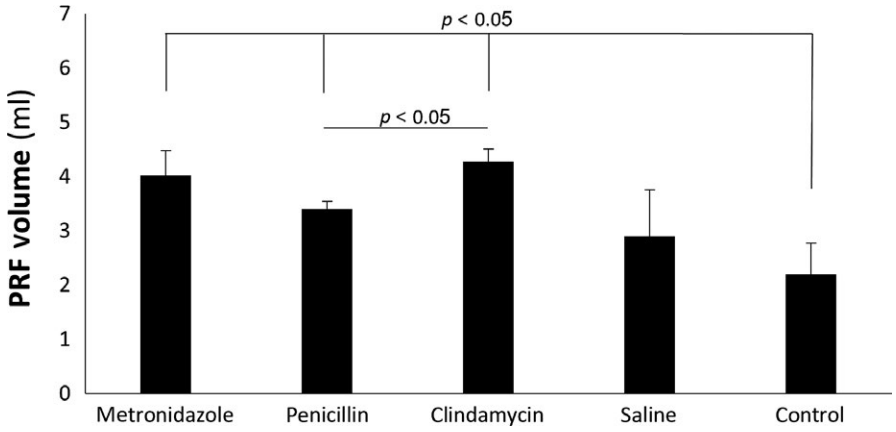

FIGURE 1 The impact of antibiotics incorporation on platelet-rich fibrin (PRF) volume. Mean volume measurement of PRFs obtained following incorporating 0.5 ml metronidazole/penicillin/clindamycin. Results are expressed as mean ± *SD*. Lines indicate groups that are statistically different

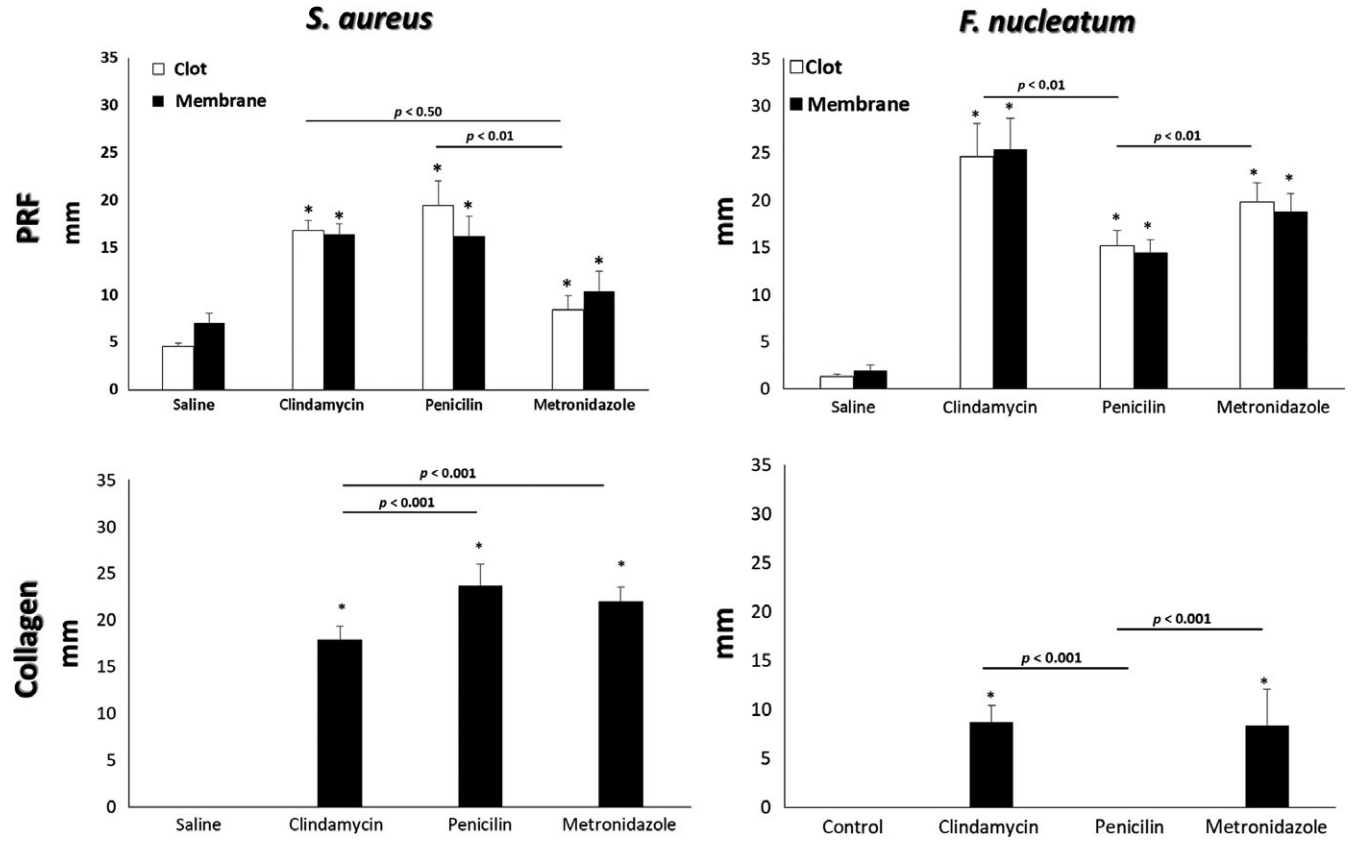

FIGURE 2 Anti-biogram test of platelet-rich fibrin (PRF) following antibiotics incorporation. *Staphylococcus aureus* and *Fusobacterium nucleatum* were grown and seeded on blood agar plates. PRF or collagen sponge following incorporating 0.5 ml metronidazole/penicillin/clindamycin were placed at the middle of the plate, and the plates were incubated in aerobic conditions for *S. aureus* and anaerobic conditions for *F. nucleatum* for 7 days. PRF was used at its clot or membrane forms. Results are expresses as bacterial inhibition radius (mean ± *SD*). Lines indicate groups that are statistically different

3.3 | Time-dependent anti-bacterial effect of PRF incorporated with antibiotics vs. natural PRF

Next, we proceeded to investigate the long-term anti-bacterial effect of the PRF incorporated with antibiotics. Since PRF at its clot or pressed (membrane) forms showed similar results, the following experiments were carried out using only the membrane form of the PRF. We had tested the anti-bacterial activity following incubation up to 4 days' post-preparation.

Platelet-rich fibrin with saline showed little bactericidal properties at all the 4 tested time intervals (Figure 3). Clindamycin and penicillin preserved their anti-*S. aureus* activity throughout the 96-hr experiment, while metronidazole showed little anti-*S. aureus* activity (similar to levels found for PRF with saline, Figure 3).

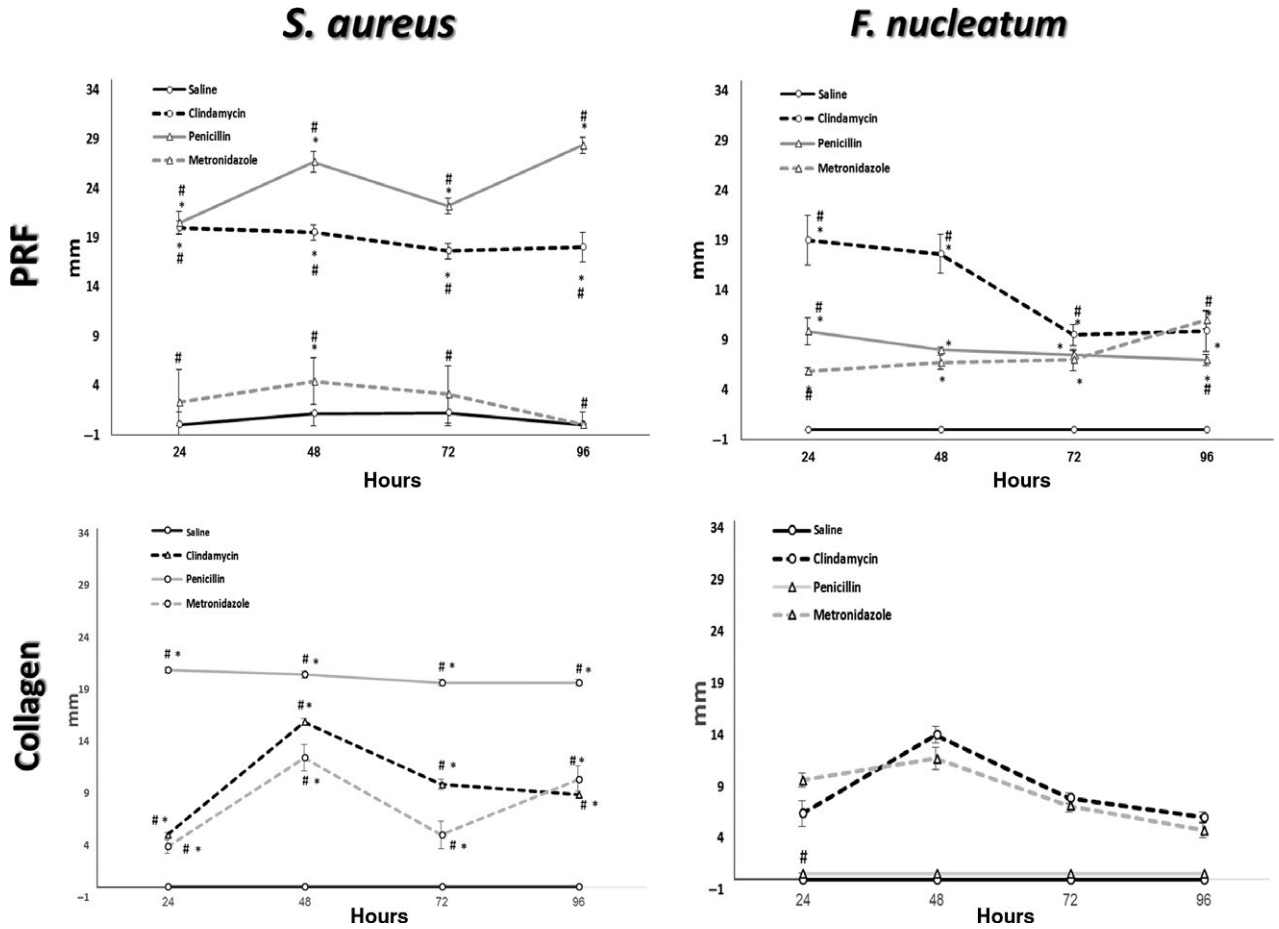

FIGURE 3 Long-term anti-biogram test of platelet-rich fibrin (PRF) following antibiotics incorporation. PRF (clot form) or collagen sponge following incorporating 0.5 ml metronidazole/penicillin/clindamycin was incubated in 10 ml PBS at 37°C for the duration of 24–96 h. *Staphylococcus aureus* and *Fusobacterium nucleatum* were grown and seeded on blood agar plates. Following incubation, PRFs and collagen sponges were placed at the middle of the plate for incubation of the plates in aerobic conditions for *S. aureus* and anaerobic conditions for *F. nucleatum*. Results are expresses as bacterial inhibition radius (mean ± *SD*). Lines indicate groups that are statistically different

Anti-*F. nucleatum* activity was observed for clindamycin throughout the experiment, despite a reduction in its potency at 72 hr (Figure 3). Both metronidazole and penicillin showed a consistent level of anti-*F. nucleatum* properties at all tested time intervals, at levels similar to that observed for clindamycin at 96 hr (Figure 3).

Collagen sponge alone did not show any bactericidal activity, and the addition of all tested antibiotics renders the sponge with anti-*S. aureus* properties at all tested time intervals. The anti-*S. aureus* activity of clindamycin was more potent using PRF than collagen sponge. Addition of penicillin to the collagen sponge showed no anti-*F. nucleatum* effect. Collagen sponge with clindamycin and metronidazole showed anti-*F. nucleatum* activity which was preserved throughout the 4 days of experiment. This activity was lower than the activity observed for PRF (Figure 3).

4 | DISCUSSION

The aim of the present study was to evaluate whether PRF incorporated with antibiotics can serve as anti-bacterial agent for the first days of healing. The addition of antibiotics to the PRF demonstrated significant inhibition of both aerobic (*S. aureus*) and anaerobic (*F. nucleatum*) growth compared to PRF alone, without a difference between the clot or pressed (membrane) forms of PRF.

The use of antibiotics, particularly in local delivery systems, may provide high doses of antibiotics to the affected tissues, exceeding the minimum inhibitory concentration by more than 1,000-fold (McLaren, 2004; Stevens, Tetsworth, Calhoun, & Mader, 2005). Such concentrations may lead to impaired wound healing and cytotoxic effect on various cells (Antoci, Adams, Hickok, Shapiro, & Parvizi, 2007). Also, some antibiotics, such as aminoglycoside, may be toxic to certain cells like in the ears and in the kidneys (Verdel, van Puijenbroek, Souverein, Leufkens, & Egberts, 2008). To make this study as practical as possible, the study used common antibiotic solutions in concentrations that are used in hospitals and clinics for intravenous administration. The present experiments indicated that the addition of 0.5 ml of all the tested antibiotic solutions to the blood did not interfere with PRF formation, and this combination showed anti-bacterial activity in vitro. Addition of greater volumes to the blood led to interference with PRF integrity.

F. nucleatum is a known periodontal pathogen with an ability to invade human gingival epithelial cells and remain viable inside host cells (Yang et al., 2015). *S. aureus* is a major cause of hospital-acquired surgical wound infections (Humphreys, 2012). Both bacteria have the ability to reside within the surrounding cells or tissues cause infection and interfere with healing (Bielecki et al., 2007). For those reasons, these pathogens were selected for the current study as the target microorganisms.

The study shows that PRF alone carries a mild inhibitory activity for the growth of *S. aureus*, and does not affect *F. nucleatum* growth. These findings cohere with other studies showing that platelet-rich plasma exhibited antimicrobial effect against *S. aureus* (Bielecki et al., 2007; Moojen et al., 2008), as well as other microbes such as *Escherichia coli* (Bielecki et al., 2007), *Klebsiella pneumonia* (Anitua et al., 2012), and *Enterococcus faecalis*, *Candida albicans and Streptococcus oralis* (Drago, Bortolin, Vassena, Taschieri, & Del Fabbro, 2013). However, this natural anti-bacterial activity of the blood product was by far lower than the effect of antibiotics.

Platelet-rich fibrin incorporated with all tested antibiotics exhibit significant inhibition of *F. nucleatum* growth at all the tested time intervals (from 0 up to 96 hr post-preparation). PRF with clindamycin or penicillin also significantly inhibited the growth of *S. aureus*. These results confirm that the antibiotics incorporated into the PRF preserved their activity for at least 4 days, suggesting it use as post-surgical slow-release anti-bacterial agent. Further, the antimicrobial activity of PRF with antibiotics was not changed by its compression to a membrane-like form, compared to the raw clot form. These results suggest that clinicians can use the PRF in both forms following addition of the antibiotics during PRF preparation.

Collagen dressing was used as a control device. The addition of antibiotics to the sponges showed anti-bacterial effect with a lesser potency compared with the PRF with antibiotics. According to our results, together with the additional benefits of PRF in wound healing, epithelization and the release of growth factors make PRF with antibiotics a suitable device as a dressing for surgical wounds. Furthermore, new modifications of PRF preparation as A-PRF showed superior traits to standard PRF (L-PRF), such as prolonged release of proteins and grown factors (Kobayashi et al., 2016), and induction of fibroblast migration, proliferation and expression of growth factors by fibroblasts (Fujioka-Kobayashi et al., 2017). The combination of antibiotics with the A-PRF may further advance its wound healing beneficial traits. Such concept was already introduced by Miron and Zhang (2018), who discussed the addition of bioactive materials with liquid PRF to produce an advanced local delivery devices.

In conclusion, the present study provides the clinician with a novel tool to control post-operative infection using modified PRF with anti-bacterial properties. Such agent can be highly important as a topical surgical tool that promotes tissue healing and prevents local infection. Moreover, such application may reduce the need for systemic antibiotic regimens. Still, the clinical translation of the present in vitro results should be taken with caution. The anti-bacterial activity of the device and the possibility that the modification may alter the L-PRF healing properties should be verified in animal and clinical studies.

CONFLICT OF INTEREST

The research is original, not under publication consideration elsewhere, and free of conflict of interest.

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