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RESEARCH ARTICLE

Cytomorphological Analysis of Liquid PRF Produced with DUO Fixed Angle Centrifuge (Process, France)

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ABSTRACT

***Background*: Liquid PRF is a second-generation injectable platelet concentrate rich in platelets, leukocytes, and fibrinogen obtained by centrifugation of autologous blood.**

***Methods*: This study aims to analyze the cellular and Fibrinogen content of various types of Liquid PRF (C-PRF liquid, A-PRF liquid, i-PRF, Liquid Fibrinogen) obtained with the use of DUO Fixed Angle Centrifuge (PRF DUO, Process for PRF, Nice, France) with Vacumed FL tubes (code 44909) and/or original S-PRF Sticky tube. An average accumulation of thrombocytes of almost 1.5 times compared to whole blood was found. Due to the high concentration of platelets, PRF-Liquids contain important growth factors for tissue regeneration.**

***Results*: In this preliminary study, we have shown that the type of Liquid PRF with a higher content of Platelets (126.3% vs. 109.5%), Monocytes (127.6% vs. 84.6%), with a sufficient content of Lymphocytes (192.9% vs. 242.1%) and Neutrophilic Granulocytes (64.6% vs. 64.8%) and Fibrinogen (67.9% vs. 87.3%), is the i-PRF (700 rpm × 5′) obtained with Vacumed FL tube (code 44909) with statistically insignificant differences compared to whole blood, while the content of Lymphocytes and Fibrinogen present in i-PRF (700 rpm × 5′) obtained with PRF-S-Sticky tube is higher. In comparison, the content of cells and Fibrinogen obtained with the two methods of preparing PRP is much lower.**

***Conclusions*: This indicates that the liquid PRF is more suitable to be used in various cases of tissue regeneration such as facial aesthetics, intra-articular injection, peri-ulcerative injection, etc., is the i-PRF (700 rpm × 5′) obtained with a Vacumed FL tube.**

Keywords: Liquid Platelet Rich Fibrin, Platelet Concentrate, PRP, Tissue Regeneration.

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1. Introduction

A liquid formulation of PRF (Platelet Rich Fibrin) can be used alone or in combination with various biomaterials. A liquid formulation of platelet-rich fibrin, termed injectable-PRF (i-PRF), was studied by Shao Z. *et al*. by centrifugation at 700 rpm for 3 minutes (60 g) without anticoagulants [1]. The data suggest that i-PRF can accelerate the proliferation of fibroblasts. Meanwhile, the same authors obtained an injectable fibrin scaffold (IFS) rich in growth factors by centrifugation at 3000 rpm for 10 minutes. This liquid scaffold contains large amounts of fibrin, which trap large numbers of platelets, white blood cells, and growth factors, and can promote proliferation, cell migration, and matrix secretion. IFS can promote the secretion of type I and type III collagen by skin fibroblasts to promote tissue repair in vivo.

Furthermore, the fibrin network scattered in the liquid scaffold favors the stable and prolonged release of growth factors for more than two weeks (up to 28 days) [2], as well as transporting stem cells. Therefore, this IFS can be used in regenerative medicine alone or as an adjunct to other biomaterials. The concentrated platelets present in

the i-PRF are responsible for the active secretion of growth factors and for inducing the requirement, proliferation, and differentiation of various cells involved in the regeneration process.

Platelet activation begins immediately upon contact with the wall of the centrifuge tube and leads to the formation of a dense fibrin network and a usable PRF clot. The slow polymerization of fibrin allows for increased entrapment of circulating (intrinsic) cytokines in the fibrin matrix. A recent study demonstrated that the reduction of the relative centrifugal force leads to a significant increase in the total number of platelets and leukocytes and the number of growth factors, indicating that the concept of low-speed centrifugation (LSCC) increases the PRF regeneration potential [3].

It is therefore pertinent to know the concentration and quantity of platelets that can be obtained and how leukocytes are concentrated in a particular liquid preparation. In this experiment, we asked ourselves the following questions:

-What concentration of thrombocytes and leukocytes could be collected within the supernatant in the different liquid PRF production protocols?

-Which protocol gives better results in terms of platelets and fibrinogen collected?

In general, after centrifugation, an average of about 3 ml of PRF was obtained from a tube containing 9 ml of blood equal to 9.1 g.

2. Materials and Methods

This study aims to find out and confirm the best tube type and the best centrifugation speed with fixed angle centrifuge DUO (PRF DUO, Process for PRF, Nice, France) to obtain liquid PRF with the highest cell content and fibrin. The main objective is to investigate whether the g-force adaptation for the above modifications on PRF liquid (C-PRF liquid, A-PRF liquid, and i-PRF), using a 41.3° fixed angle centrifuge and with different types of the test tube, has some influence on their characteristics in terms of morphology and cellular content, as well as on the content of fibrinogen precursor of the fibrin reticulum. A comparison was also performed with PRP (Platelet Rich Plasma) produced at 2200 rpm × 20′ with PRP BioReb Gel (Centrifuge Hettich EBA 200 angle of 33°) with single centrifugation and with double centrifugation according to the procedure recommended by Rattanasuwan *et al*. [4].

The centrifugation of the PRF in the negative pressure vacuum collection tube in Plastic (PET) Vacumed FL (code 44909) and with the original S-PRF Sticky test tube (recommended by the manufacturing company Process, France) allows preparing the PRF in liquid form and to use it in an injectable way. Liquid PRF can be used in various cases of tissue regeneration such as facial aesthetics, intra-articular injection, peri-ulcerative injection, etc.

C-PRF liquid (RCF*clot* = 525 g; RCF*max* = 700 g; RCF*min* = 280 g) sec. Miron [5], [6] or other types of liquid PRF resulted after post-centrifugation sampling of the resulting liquid, with a sterile syringe and 18 g needle, which can be injected or even solidified into clots and membranes. In this study, the content of fibrin and cells (neutrophil granulocytes, monocytes, lymphocytes, and platelets) was evaluated both with a blood count and with an optical microscope at 10–100 × magnification with staining. Methylene blue and May-Grûnwald stains were performed to stain the PRF liquid smears to confirm the cell content determined by hemocytometry.

Some researchers are concerned about a possible health hazard when glass vacuum blood collection tubes with silica activators are used. O'Connell [7] described the inevitable contact with silica. For this, we have only used silica-free tubes.

The study did not require local ethics committee approval as no foreign substance was administered, and in any case, each patient was included in the study after obtaining written consent and then informed about the procedures.

The haematological samples of 45 subjects in apparent well-being aged between 35 and 101 years were examined, of which 23 were male and 22 were female.

2.1. *Inclusion Criteria*

No intake of anticoagulants or functional equivalents for one month before enrollment and platelet count in the range of 150,000 to 450,000/μL and coagulation index determined as normal [prothrombin time (PT value) between 11 and 16 seconds]. Hemoglobin concentration >9 mg/dL, serum protein concentration >6 g/dL, and serum albumin >3 g/dL.

Absolute contraindications for PRF production include S. from platelet dysfunction, critical thrombocytopenia, hemodynamic instability, and sepsis. Relative contraindications include heavy smokers, drug and alcohol users, patients with chronic liver disease, severe metabolic or systemic disorders, patients with cancer of hematopoietic origin, with low hemoglobin (<10 g/mL) or platelet count (<1.2 × 10^5/μL). Also, patients taking NSAIDs, prednisolone >20 mg/day, and anticoagulant therapy were excluded.

2.2. *Blood Chemistry Procedures*

Liquid PRF is a second-generation platelet concentrate, which includes various growth factors, thrombocytes, leukocytes, $CD34^+$ stem cells, and a fibrinous matrix [8]. A quantity of 1.5 ml of liquid PRF was placed in a test tube with EDTA K3E 5.4 mg to perform a blood count and, using a HECO 5 hematology analyzer (Seac Radim Company), and the concentration of Monocytes, Granulocytes Neutrophils, Lymphocytes and Platelets (the average values obtained for each type of liquid PRF produced have been reported).

Fibrinogen was measured by an immunoturbidimetric test with ACL 3000 (Beckman Instrumentation Laboratory). We, therefore, wanted to evaluate the content of Fibrinogen, Platelets, Monocytes, Neutrophil Granulocytes, and Lymphocytes, in the peripheral blood and, again by carrying out a blood count, on the liquid PRF of various types [A-PRF liquid (1300 rpm × 5′); i-PRF (700 rpm × 5′); i-PRF (3300 × 3′); C-PRF (2500 rpm × 8′) [5]; Liquid fibrinogen (2700 rpm × 3′) [9]–[11] obtained using two different types of tubes for centrifugation of blood collected from patients (Vacumed

Fig. 1. Incubator used to keep the tubes at a constant temperature of 37 °C.

FL and Verdi S-PRF sticky tubes) heated in an incubator at 37 °C (Fig. 1) to stimulate body temperature as much as possible.

We also provided for the production of PRP produced at 2200 rpm × 20′ with BioReb Gel PRP Kit (C.O. Biotechology S.r.l., Na, Italy) according to the manufacturer's indications on 25 patients and for the evaluation of Monocytes, Neutrophil Granulocytes, Lymphocytes, Platelets with blood count and fibrinogen [4], [9]. PRP was also prepared as previously described by Rattanasuwan *et al*. [4] with minor modifications. Briefly, blood was centrifuged in a cell separator (Hettich EBA 200) at 1,300 rpm (rpm) for 10 min after placing it in a BioReb Gel PRP Kit tube. The blood was then separated into a lower region of red blood cells and an upper region of straw-colored plasma. The straw-colored plasma was taken and centrifuged again at 2000 rpm for 10 min in a Vacumed FL test tube. The result of the second centrifugation consisted of a light-yellow upper portion of supernatant serum and a red-shaded lower portion. The top of the serum was discarded and approximately 1.5 mL of concentrated serum and platelets remained in the tube. The resulting PRP should have according to Rattanasuwan *et al*. [4] an increase of 331% compared to the baseline concentration of platelets and in any case not less than 200% [9].

2.3. Cytological Procedures for Light Microscopy

PRF-Liquid staining shows scattered fibrous reticular structures and there are large quantities of white blood cells and platelets (Fig. 2). Smears of liquid PRF were prepared on microscope slides and stained with Methylene Blue (30'), May-Grûnwald (3'), or Toluidine Blue (30').

All smeared slides were washed with demineralized water after staining and immediately analyzed under an optical microscope.

2.4. Histomorphometric Analysis

Cytometric analysis was performed by the examiner blinded to the centrifugation technique used. The stained slides were examined and images of three different areas of each section (0.348 mm^2) were taken through an optical microscope with an integrated camera, in groups at an original magnification of 10, 20, 40, 60, 100 × immersion (Optika, B-150D-BRPL, Optika S.r.l. Italy). The digital images were saved on a computer. The AmScope MD 500-CK 5.0 mp (United Scope, LLC, NNL), Vers.2022 software was used for the histomorphometric analysis.

2.5. Statistical Measurements and Analyses

Data of continuous variables obeying the normal distribution were expressed as "Mean ± Standard Deviation" ($x \pm sd$), "Standard Error", and "Median". As regards the statistical significance of the measurement of the cytological and fibrinogen values detected in the PRF-Liquid, the differences between the groups were calculated with the Student's *T*-test for repeated measures for parametric variables and with the signed *U-test Mann-Whitney* for non-normally distributed data. Each value of $p \leq 0.05$ was considered statistically significant, $p \leq 0.005$ was highly significant, and $p \geq 0.05$ was considered non-significant.

Data were analyzed using version 6.0 of the Santon-Glantz 2007 Statistics for Biomedical Disciplines package.

3. Results

The procedure was well tolerated by all subjects examined. The mean age was 65.07 ± 14.24 aa, E.S.: 2.12; Median 66 y. No significant differences were found in the baseline hematologic comparison of test subjects, who had a mean RBC concentration of 4.6 × 10^6/mL (±1.1; 95% CI) (p = 0.34)(range: 4–5.5 × 10^6/ mL), WBC of 5.1 × 10^3/mL (±0.37; 95% C.I.)(p = 0.24)(range: 4.5–8.5 × 10^3/mL) and a Mean Platelet Count of 296.8 × 10^3/mL (±15.3; I.C.95%) (p = 0.15) (range:150–400 × 10^3/mL). The complete blood count performed on the various types of PRF-Liquid generally shows that the IFS includes the content of white blood cells >25% and platelets >50% compared to whole blood. Specifically, the white blood cells contained in the liquid scaffolds were almost all lymphocytes, few were neutrophil granulocytes, and few were monocytes. The quantity of liquid PRF obtained from all PRF-Liquids both with S-PRF Sticky and Vacumed FL test tubes is approximately 2–3 ml. We have also tried to heat the test tubes used for centrifugation before sampling and, afterward, to obtain a greater quantity of PRF-Liquid and also a clot from it, using an incubator at 37 °C (Fig. 1) to be favored in this way, the formation of the fibrin clot, by polymerization from the fibrinogen, under the influence of the physiologically available thrombin.

3.1. Growth Factors EGF and VEGF

Zwittnig *et al*. [10] evaluated growth factors (of particular interest to us are EGF and VEGF) with a centrifugation process lasting 8 min, with 1200 rpm and a relative centrifugal force (RCF) of 177 g. The liquid and solid PRFs produced were used to analyze the release of five growth factors over 10 days.

Particularly interesting for our studies due to their effect on angiogenesis was the evolution of the release of EGF and VEGF in the comparison between Solid and Liquid PRF shown in Fig. 3.

After 1 hour, an EGF (Epidermal Growth Factor) concentration of 71 pg/mL was measured in the PRF-Liquid.

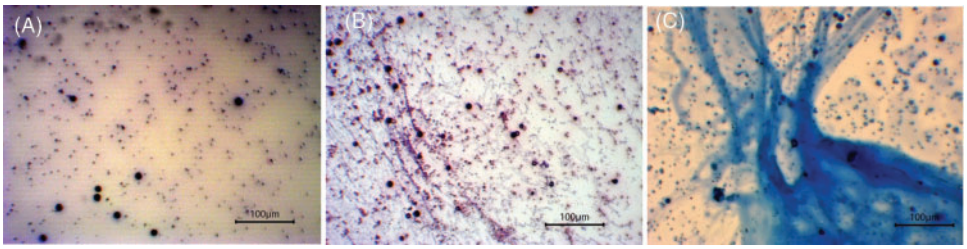


Fig. 2. (A) i-PRF 700 rpm × 5′ with Toluidine Blue, (B) May-Grûnwald and (C) Methylene Blue staining, respectively. A large number of platelets and many leukocytes (lymphocytes) are evident. Input × 10 (scale bar 100 microns).

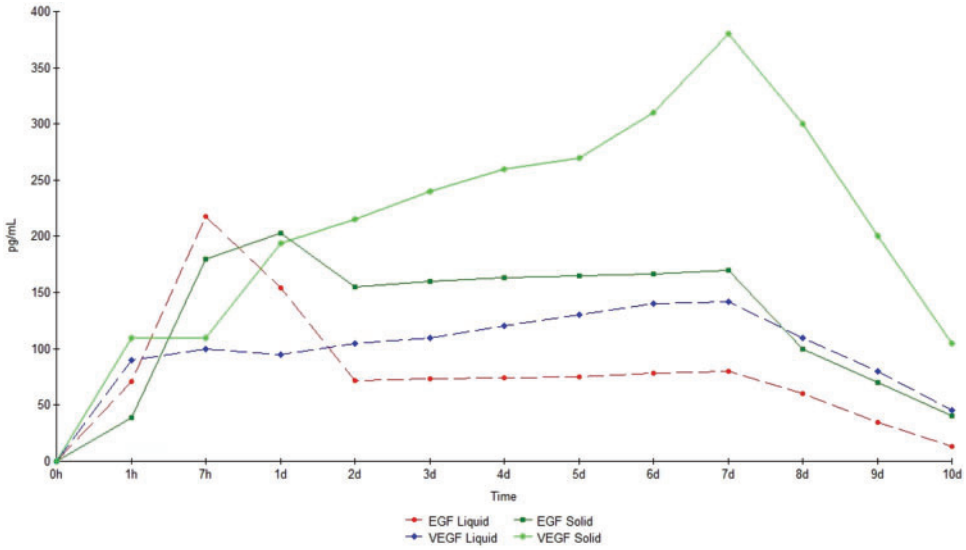


Fig. 3. The ratio between EFG and VEGF release between Solid PRF and Liquid PRF (from Zwittnig, 2022 modified) [10].

The highest value of 218 pg/mL was highlighted after 7 hours. After a steep increase during the first 24 hours, by day 2, EGF values decreased from 154 to 72 pg/mL and finally to 13 pg/mL on day 10. The release of VEGF (Vasoendothelial Growth Factor) was consistently around 90–100 pg/mL over the first 2 days. The highest VEGF release in liquid PRF was observed on day 7 (142 pg/mL). Until day 10, the value dropped to 45 pg/mL (Fig. 3).

Solid PRF released 39 pg/mL of EGF after 1 hour but increased to a plateau of 203 pg/mL from the 7th hour to day 7. On day 10, it decreased to 40 pg/mL. VEGF values started from 110 pg/mL after 1 hour and 110 pg/mL after 7 hours. They increased to 194 pg/ml on day 1 and 215 pg/ml on day 2. Their maximum value was 380 pg/ml on day 7. On day 10, VEGF was 105 pg/ml (Fig. 3).

3.2. *Cytological Study in Light Microscopy*

Both C-PRF liquid 2500 rpm × 8′ obtained in the Vacumed FL tube and in the S-PRF Sticky tube (RCF_{clot} = 525 g; RCF_{max} = 700 g; RCF_{min} = 280 g) had a cellular presence highlighted with Methylene Blue staining ed May-Grûnwald in the low-power field (ingr. 10×, 20×) (Figs. 4A–4H, respectively). A conformal fibrin network structure was not observed in any of the samples examined.

Blood-derived cellular elements were identified in various concentrations depending on the centrifuge tube used (Fig. 4).

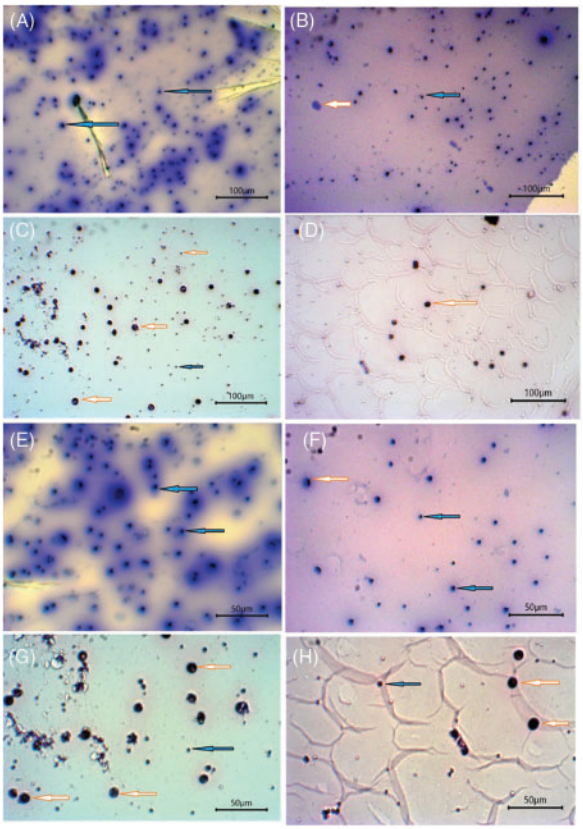
Higher concentrations of lymphocytes (97.85% vs. 66.17%) and fibrinogen (88.17% vs. 42.8%) compared to whole blood were found in C-PRF liquid obtained with Vacumed FL tube compared to those with original S-PRF Sticky (Tables I and II) while Platelets (20.98% vs. 35.21%) are higher with the use of S-PRF Sticky tubes and are specifically reported in the captions of Fig. 4 and the graphs contained in Fig. 5.

The Monocytes content which is particularly interesting for us for use in angiogenesis is very low in this particular type of liquid PRF, whatever the type of centrifuge tube used (9.76% vs. 2.7%).

In particular, mononuclear cells (monocytes and lymphocytes):

- *modulate the inflammatory process* through the local production of a large number of cytokines and other specific substances which initiate the tissue healing process;
- *stimulate the formation of new blood vessels* through the release of angiogenic cytokines and growth factors in the microenvironment of tissue damage (paracrine effect), contributing to the vascular remodeling of the tissue to be regenerated.
- *stimulate stem cells and local progenitor cells* to promote reparative processes [11].

Chronic low-grade inflammation within the vascular wall is associated with monocyte infiltration. The maturation of monocytes into macrophages is accompanied by the production of cytokines and growth factors. A large percentage of circulating endothelial progenitor cells (EPCs) are of monocytic origin. Monocytes are therefore widely involved in the formation of vasa vasorum mediated by VEGF [12].



Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 4. (A) C-PRF liquid 2500 rpm × 8′ (Vacumed FL test tube), col. Methylene Blue, ingr. 10 x, Platelets are almost exclusively evident, few leukocytes are present; (B) C-PRF liquid 2500 rpm × 8′ (S-PRF Sticky test tube) col.Methylene Blue, ingr.10 ×, Fewer platelets and leukocytes are highlighted compared to A; (C) C-PRF liquid 2500 rpm × 8′ (Vacumed FL tube) col. May-Grûnwald ingr. 10x, WBCs (some granulocytes and many lymphocytes) and many platelets are seen; (D) C-PRF liquid 2500 rpm × 8′ (S-PRF Sticky tube) col. May-Grûnwald ingr. 10 x, Many platelets, few lymphocytes are evident; (E) C-PRF liquid 2500 rpm × 8′ (Vacumed FL test tube) col.Methylene Blue, ingr.20 ×, Some granulocytes and lymphocytes and many platelets are highlighted; (F) C-PRF liquid 2500 rpm × 8′ (S-PRF Sticky test tube) col.Methylene Blue, ingr.20 x, Only few platelets are highlighted; (G) C-PRF liquid 2500 rpm × 8′ (Vacumed FL tube) col. May-Grûnwald, ingr.20 ×, Some granulocytes and lymphocytes and few platelets are highlighted; (H) C-PRF liquid 2500 rpm × 8′ (S-PRF Sticky tube) col. May-Grûnwald, ingr.20 ×, Very few platelets and few lymphocytes are highlighted (A, B, C, D scale bar 100 µm); (E, F, G, H scale bar 50 µm).

Advanced-PRF liquid 1300 rpm × 5′ was obtained in a Vacumed FL tube and the S-PRF Sticky tube (RCF_{clot} = 142 g; RCF_{max} = 189 g; RCF_{min} = 66 g). A cellular presence was found highlighted with Methylene Blue and May-Grûnwald staining with low magnification field of (10×, 20×, 40× and 100× immersion) in Figs. 6A–6H, respectively, with the use of the S-PRF Sticky tube. A conformal fibrin network structure was not observed in any of the examined samples. The same Figs. 6E'–6G' show the cellular contents present in the squeezing liquid of A-PRF liquid coagulated in the Vacumed FL test tube at 37 °C and compressed in the PRF Box for 2 minutes, colored with Methylene and May-Grûnwald, and few cellular elements are found except for the few platelets present.

Values found in A-PRF liquid 1300 rpm × 5′ (S-PRF Sticky) are presented in Table III.

Even with the use of the Vacumed FL tube in the production of A-PRF Liquid and with a mixed Toluidine Blue/May-Grûnwald stain (Fig. 7), a shaped fibrin network was not observed, while cellular elements derived blood is present in various concentrations.

Higher concentrations of Platelets (115.58% vs. 97.74%), Monocytes (54.9% vs. 33.3%), and Fibrinogen (88.01% vs. 87.54%) compared to whole blood were found in A-PRF liquid obtained with Vacumed FL tubes compared to those with original S-PRF Sticky (Tables III and IV). The Lymphocytes (193.10% vs. 182.32%) are higher with the use of S-PRF Sticky tubes and are extensively reported in the captions of Fig. 6, while those with the tube Vacumed FL are shown in the captions of Fig. 7 and the graphs in Fig. 5.

Values found in A-PRF liquid 1300 rpm × 5′ (Vacumed FL) are presented in Table IV.

In the i-PRF 700 rpm × 5′ obtained in Vacumed FL test tube and in S-PRF Sticky test tube ($RCF_{coagulum}$ = 38 g; RCF_{max} = 55 g; RCF_{min} = 22 g). A cellular presence highlighted with the Methylene Blue and May-Grûnwald staining in the field at low power (magn. 10 ×, 20 ×, 40 × and 60 ×) and shown in Figs. 8A–8H, respectively, with the use of a Vacumed FL tube. Again, a well-conformed fibrin network structure was not observed in any of the samples examined, except for some thin filaments.

Values found in i-PRF 700 rpm × 5′ (Vacumed FL) are presented in Table V.

Even with the use of the S-PRF Sticky test tube with Methylene Blue and May-Grûnwald staining (Figs. 9A–9H, respectively) (ingr.10, 20, 40, 60 ×) a fibrin network shaped in i-PRF liquid (700 rpm × 5′) was not observed, while cellular elements of blood derivation were identified, present in different concentrations and reported in Table VI.

Values found in i-PRF liquid 700 rpm × 5′ (Green S-PRF sticky test tube) are presented in Table VI.

Higher concentrations of Platelets (126.27% vs. 109.49%) and Monocytes (127.59% vs. 84.62%) compared to whole blood were found in i-PRF liquid obtained with Vacumed FL tube rather than with original S-PRF Sticky (Tables V and VI) while Lymphocytes (242.12% vs. 192.99%) and Fibrinogen (87.3% vs. 67.97%) are higher with the use of S-PRF Sticky tubes and are specifically reported in the captions of Fig. 9, while those with the Vacumed FL are illustrated in the captions of Fig. 8 and the graphs in Fig. 5.

Particularly interesting was the concentration of Monocytes present in i-PRF 700 rpm × 5′ for possible use in vascular regeneration [11] obtained with the Vacumed FL test tube (127.59%) compared to the S-PRF Sticky (84.62%). Indeed, in vivo, monocytes differentiate into ECs (Endothelial Cells) and are incorporated into blood vessels [13].

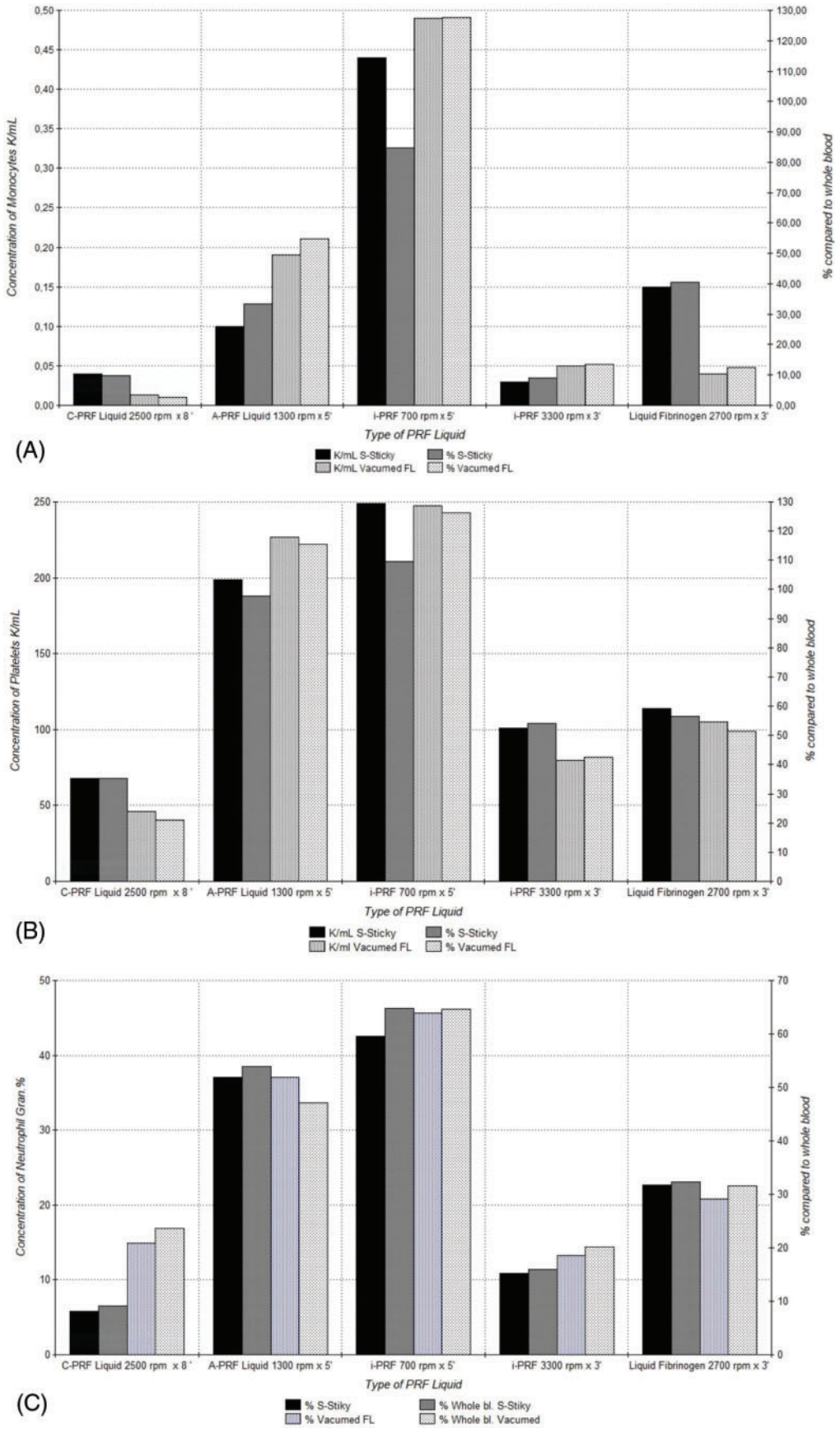


Fig. 5. Graphical comparison between the content of Monocytes (A), Platelets (B), Neutrophil Granulocytes (C).

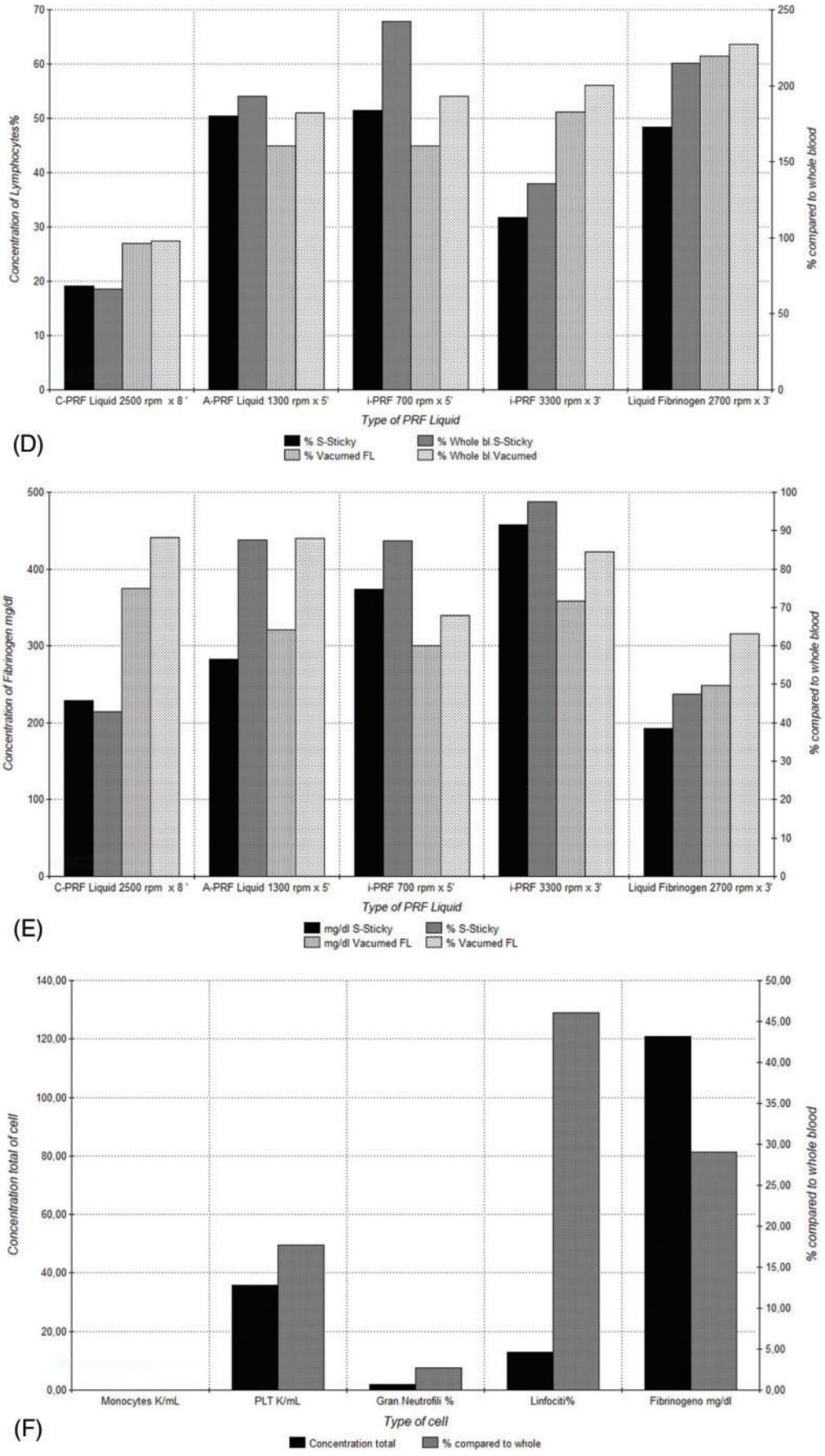


Fig. 5. Lymphocytes (D) and Fibrinogen (E) in the various types of Liquid PRF produced with original S-PRF Sticky tube and Vacumed FL in absolute values (mg/dl) and as a relative percentage in relation to the initial content in whole blood.
In (F) the cellular content found in the PRP.

TABLE I: Comparison between Fibrinogen Values and Cells in C-PRF Liquid 2500 × 8′ in Vacumed FL Tube and Whole Blood

Type	C-PRF liquid vacumed FL (2500 rpm × 8′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average±D.S.	Standard Error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.013 ± 0.035	0.009	0.0	0.49 ± 0.19	0.05	0.50	0.000∗	0.000∗	2.70%
PLT K/μL	46.1 ± 37.5	9.68	37.0	219.9 ± 42.0	10.86	218.0	0.000∗	0.000∗	20.98%
Neutr.Gran. %	14.87 ± 20.58	5.31	0.0	62.9 ± 6.5	1.67	60.4	0.000∗	0.000∗	23.60%
Lymphocytes %	26.96 ± 32.8	8.48	0.0	27.5 ± 5.5	1.42	27.2	0.094	0.361	97.85%
Fibrinogen mg/dl	374.8 ± 155.4	40.1	351.0	425.1 ± 116.8	30.1	401.0	0.247	0.419	88.17%

Note: ∗ Statistically significant difference.

TABLE II: Comparison between Fibrinogen Values and Cells in C-PRF Liquid 2500 × 8′ in S-PRF Sticky Tube and Whole Blood

Type	C-PRF liquid S-PRF sticky (2500 rpm × 8′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.04 ± 0.13	0.04	0.0	0.41 ± 0.17	0.055	0.35	0.000∗	0.000∗	9.76%
PLT K/μL	68.0 ± 44.8	14.18	58.0	193.1 ± 39.9	12.62	195.0	0.000∗	0.000∗	35.21%
Neutr.Gran. %	5.78 ± 12.1	3.82	0.0	63.4 ± 7.5	2.36	62.9	0.000∗	0.000∗	9.12%
Lymphocytes %	19.1 ± 30.7	9.72	0.0	28.8 ± 4.4	1.39	28.5	0.366	0.140	66.17%
Fibrinogen mg/dl	228.5 ± 252.3	79.8	164.0	533.9 ± 150.8	47.7	504.5	0.008∗	0.009∗	42.8%

Note: ∗ Statistically significant difference.

Also, in the i-PRF 3300 rpm × 3′ obtained in Vacumed FL tube and in S-PRF Sticky tube (RCF $_{coagulum}$ = 765 g; RCF $_{max}$ = 1008 g; RCF $_{min}$ = 403 g) a cellular presence was found highlighted with the Methylene Blue and May-Grûnwald low power field staining (ingr. 10 ×, 20 ×) in Figs. 10A–10D, respectively, for the Vacumed FL tube. Again, a well-conformed fibrin network structure was not observed in any of the samples examined, except for some thin filaments. Even with the use of the S-PRF Sticky tube with Methylene Blue and May-Grûnwald staining (Figs. 10A'–10D', respectively) (ingr.10, 20 ×) no hinted fibrin network was observed, while cellular elements of blood derivation were identified, present in different concentrations and reported in Tables VII and VIII.

Higher concentrations of Platelets (54.02% vs. 42.52%), and Fibrinogen (97.6% vs. 84.44%) compared to whole blood were found in i-PRF liquid 3300 rpm × 3′ obtained with the original S-PRF Sticky tube compared to Vacumed ones FL (Tables VII and VIII) while Lymphocytes (200.37% vs. 135.43%), Neutrophil Granulocytes (20.10% vs. 15.87%) and Monocytes (13.56% vs. 9.09%) are higher with the use of Vacumed FL tubes and are reported specifically in the captions of Fig. 10 and the graphs in Fig. 5.

The platelet concentrate defined as Liquid Fibrinogen 2700 rpm × 3′ [14] obtained in a Vacumed FL tube and S-PRF Sticky tube (RCF $_{clot}$ = 408 g; RCF $_{max}$ = 653 g; RCF $_{min}$ = 326 g) was also, and a cellular presence was found highlighted with Methylene Blue and May-Grûnwald staining in the field at low power (ingr. 10 ×, 20 ×) in Figs. 11A–11D, respectively, for the Vacumed FL tube. Even in this case, a well-conformed fibrin network structure was not observed in any of the samples examined, except for some thin filaments highlighted by May-Grûnwald staining. Also, with the use of the S-PRF Sticky tube and staining with Methylene Blue and May-Grûnwald (Figs. 11A'–11D', respectively) (ingr.10, 20 ×), no shaped fibrin network was observed, while cellular elements of blood derivation present in different concentrations were identified, which are reported in Tables IX and X.

Higher concentrations of Platelets (56.43% vs. 51.47%), Monocytes (40.54% vs. 12.55%) and Neutrophil Granulocytes (32.25% vs. 31.61%) compared to whole blood were found in liquid fibrinogen obtained with original S-PRF Sticky tube rather than with Vacumed FL (Tables IX and X), while Fibrinogen (63.1% vs. 47.36%) and Lymphocytes (227.19% vs. 215.02%) are higher with the use of Vacumed FL tubes and are specifically reported in the captions of Fig. 11 and the following graphs in Fig. 5.

In PRP (Platelet Rich Plasma) extracted in 25 patients (2200 rpm, 1147 g × 20′) with 15 ml tube BioReb Base (Biodevice & Advanced Materials S.r.l. Napoli, Italy) with separator gel and anticoagulant (ACD-A: Anticoagulant Citrate Dextrose Solution, Solution A, USP) with a HETTICH EBA200 centrifuge at a fixed angle of 33°), but also in that produced with double centrifugation [4] a cellular presence was found highlighted with the Methylene Blue and May-Grûnwald low-power field staining (ingr. 10×, 20×, 40×) in Figs. 12A–12C and 12A'–12C', respectively. Again, a well-established fibrin network structure was not observed in any of the samples examined. A low concentration of platelets was found compared to whole blood

TABLE III: Comparison between Fibrinogen Values and Cells in A-PRF Liquid 1300 × 5′ in S-PRF Sticky Tube and Whole Blood

Type	A-PRF liquid S-PRF sticky (1300 rpm × 5′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.10 ± 0.15	0.047	0.0	0.30 ± 0.12	0.039	0.30	0.001∗	0.012∗	33.33%
PLT K/μL	198.8 ± 116.3	36.77	198.5	203.4 ± 58.1	18.37	205.0	0.901	0.910	97.74%
Neutr.Gran. %	37.06 ± 12.6	3.99	37.4	68.7 ± 8.0	2.53	67.6	0.000∗	0.000∗	53.97%
Lymphocytes %	50.45 ± 13.8	4.36	43.5	26.1 ± 6.4	2.02	27.5	0.000∗	0.000∗	193.10%
Fibrinogen mg/dl	283.1 ± 136.5	43.2	336.0	323.4 ± 85.2	26.9	323.5	0.278	0.597	87.54%

Note: ∗ Statistically significant difference.

TABLE IV: Comparison between Fibrinogen Values and Cells in A-PRF Liquid 1300 × 5′ in Vacumed FL Tube and Whole Blood

Type	A-PRF liquid vacumed FL (1300 rpm × 5′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.19 ± 0.22	0.058	0.2	0.34 ± 0.19	0.051	0.30	0.049∗	0.007∗	54.90%
PLT K/μL	226.5 ± 112.4	29.03	239.0	195.9 ± 49.5	12.79	182.0	0.331	0.221	115.58%
Neutr.Gran. %	37.06 ± 12.6	3.99	37.4	69.6 ± 7.3	1.88	68.1	0.000∗	0.000∗	47.12%
Lymphocytes %	44.97 ± 23.5	6.08	48.8	24.7 ± 6.6	1.704	27.7	0.001∗	0.002∗	182.32%
Fibrinogen mg/dl	320.5 ± 133.2	34.4	332.0	364.1 ± 98.3	25.4	323.5	0.295	0.384	88.01%

Note: ∗ Statistically significant difference.

TABLE V: Comparison between Fibrinogen Values and Cells in i-PRF Liquid 700 × 5′ in Vacumed FL Tube and Whole Blood

Type	i-PRF vacumed FL (700 rpm × 5′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.49 ± 0.23	0.060	0.60	0.387 ± 0.20	0.052	0.30	0.088	0.206	127.59%
PLT K/μL	247.4 ± 130.8	33.78	253.0	195.9 ± 72.9	18.83	180.0	0.070	0.290	126.27%
Neutr.Gran. %	45.65 ± 10.9	2.80	44.8	70.6 ± 6.4	1.65	71.0	0.000∗	0.000∗	64.63%
Lymphocytes %	44.94 ± 10.8	2.78	46.3	23.3 ± 7.8	2.01	21.4	0.006∗	0.000∗	192.99%
Fibrinogen mg/dl	300.3 ± 218.4	72.8	330.0	441.9 ± 152.3	50.8	387.0	0.107	0.158	67.97%

Note: ∗ Statistically significant difference.

TABLE VI: Comparison between Fibrinogen Values and Cells in i-PRF Liquid 700 × 5′ in S-PRF Sticky Tube and Whole Blood

Type	i-PRF S-PRF sticky (700 rpm × 5′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.44 ± 0.28	0.089	0.40	0.520 ± 0.17	0.053	0.55	0.466	0.344	84.62%
PLT K/μL	249.1 ± 153.3	48.47	234.5	227.5 ± 95.5	30.19	206.5	0.427	0.678	109.49%
Neutr.Gran. %	42.54 ± 11.2	3.54	43.1	65.6 ± 22.6	7.14	70.1	0.008∗	0.004∗	64.84%
Lymphocytes %	51.41 ± 9.9	3.29	50.0	21.2 ± 9.97	3.33	20.3	0.000∗	0.000∗	242.12%
Fibrinogen mg/dl	373.3 ± 263.4	83.3	330.0	427.6 ± 200.2	63.3	369.0	0.279	0.385	87.3%

Note: ∗ Statistically significant difference.

TABLE VII: Comparison between Fibrinogen Values and Cells in i-PRF Liquid 3300 × 3′ in S-PRF Sticky Tube and Whole Blood

Type	i-PRF S-PRF sticky (3300 rpm × 3′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.03 ± 0.067	0.021	0.00	0.330 ± 0.095	0.030	0.30	0.000∗	0.000∗	9.09%
PLT K/μL	100.7 ± 55.2	17.47	99.0	186.4 ± 36.7	11.60	187.0	0.000∗	0.003∗	54.02%
Neutr.Gran. %	10.90 ± 13.4	4.25	5.5	68.8 ± 10.2	3.23	70.10	0.000∗	0.000∗	15.87%
Lymphocytes %	31.80 ± 34.3	10.85	25.0	23.5 ± 8.44	2.67	21.55	0.466	0.970	135.43%
Fibrinogen mg/dl	457.4 ± 134.5	42.52	402.0	468.6 ± 103.7	32.8	430.5	0.837	0.364	97.6%

Note: ∗ Statistically significant difference.

TABLE VIII: Comparison between Fibrinogen Values and Cells in i-PRF Liquid 3300 × 3′ in Vacumed FL Tube and Whole Blood

Type	i-PRF vacumed FL (3300 rpm × 3′)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average±D.S.	Standard Error	Median			
Monocytes K/μL	0.05 ± 0.1	0.027	0.0	0.39 ± 0.15	0.038	0.40	0.000∗	0.000∗	13.56%
PLT K/μL	79.9 ± 67.8	17.5	56.0	188.0± 36.1	9.32	191.0	0.000∗	0.000∗	42.52%
Neutr.Gran. %	13.3 ± 14.6	3.76	10.0	66.2 ± 78.0	3.04	69.0	0.015∗	0.000∗	20.10%
Lymphocytes %	51.2 ± 34.7	8.96	60.0	25.6 ± 11.3	2.92	22.1	0.011∗	0.038∗	200.37%
Fibrinogen mg/dl	358.3 ± 184.3	47.6	351.0	456.0 ± 122.8	31.7	416.0	0.099	0.147	84.44%

Note: ∗ Statistically significant difference.

TABLE IX: Comparison between Fibrinogen Values and Cells in Fibrinogen Liquid 2700 × 3′ in S-PRF Sticky Tube and Whole Blood

Type	Fibrinogen liquid sticky (2700 rpm × 3′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.15 ± 0.31	0.097	0.05	0.370 ± 0.177	0.055	0.30	0.067	0.004∗	40.54%
PLT K/μL	114.1 ± 52.2	16.5	100.0	202.2 ± 44.7	14.14	192.0	0.000∗	0.005∗	56.43%
Neutr.Gran. %	22.70 ± 22.0	6.95	15.0	70.38 ± 4.2	1.33	70.50	0.000∗	0.000∗	32.25%
Lymphocytes %	48.40 ± 31.9	10.12	59.0	22.5 ± 3.49	1.10	23.60	0.020∗	0.034∗	215.02%
Fibrinogen mg/dl	192.9 ± 222.4	70.32	99.0	397.3 ± 136.7	43.2	369.0	0.023∗	0.031∗	47.36%

Note: ∗ Statistically significant difference.

TABLE X: Comparison between Fibrinogen Values and Cells in Fibrinogen Liquid 2700 × 3′ in Vacumed FL Tube and Whole Blood

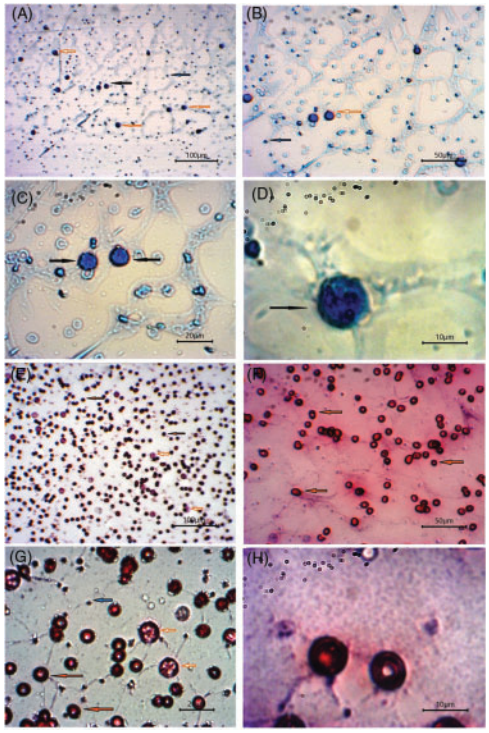
Type	Fibrinogen vacumed FL (2700 rpm × 3′)			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.04 ± 0.063	0.016	0.00	0.319 ± 0.147	0.052	0.30	0.000∗	0.000∗	12.55%
PLT K/μL	105.1 ± 74.8	18.7	196.0	202.2 ± 37.1	9.28	216.0	0.000∗	0.000∗	51.47%
Neutr.Gran. %	20.79 ± 22.4	5.6	14.0	65.8 ± 8.6	2.15	68.50	0.000∗	0.000∗	31.61%
Lymphocytes %	61.5 ± 26.5	7.0	71.20	27.1 ± 8.14	2.0	23.85	0.000∗	0.000∗	227.19%
Fibrinogen mg/dl	248.7 ± 168.3	42.07	302.5	349.2 ± 126.1	31.53	363.50	0.010∗	0.022∗	63.10%

Note: ∗ Statistically significant difference.

TABLE XI: Comparison between Fibrinogen Values and Cells in PRP 2200 × 20′ in PRP BioReb Gel Tube and With Double Centrifugation and Whole Blood [4]

Type	PRP (PRP BioReb gel (2200 rpm × 20′) and double centrifugation			Blood			*t-test* of student	Test *U* of Mann-Whitney	Restrained %
	Average ± D.S.	Standard error	Median	Average ± D.S.	Standard error	Median			
Monocytes K/μL	0.0 ± 0.0	0.0	0.0	0.33 ± 0.11	0.028	0.3	0.000∗	0.000∗	0.0%
PLT K/μL	35.9 ± 62,4	1.96	10.0	202.9 ± 54.2	14.32	215.0	0.000∗	0.000∗	17.70%
Neutr.Gran. %	1.8 ± 6,27	1.47	0.0	66.1 ± 5.5	1.32	67.0	0.000∗	0.000∗	2.72%
Lymphocytes %	12.9 ± 30.3	4.40	0.0	28.0 ± 5.3	1.38	26.8	0.000∗	0.000∗	46.1%
Fibrinogen mg/dl	120.9 ± 120.9	49.3	0.0	417.4 ± 125.3	32.98	374.0	0.001∗	0.002∗	29.0%

Note: ∗ Statistically significant difference.



Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 6. (A) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr.10 ×, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 100 μm); (B) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr. 20 ×, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 50 μm); (C) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr.40 ×, Many Platelets and Neutrophilic Granulocytes (Methylene Blue) are highlighted (scale bar 20 μm); (D) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr.100 ×, A Neutrophilic Granulocyte (Methylene Blue) is highlighted (scale bar 10 μm).

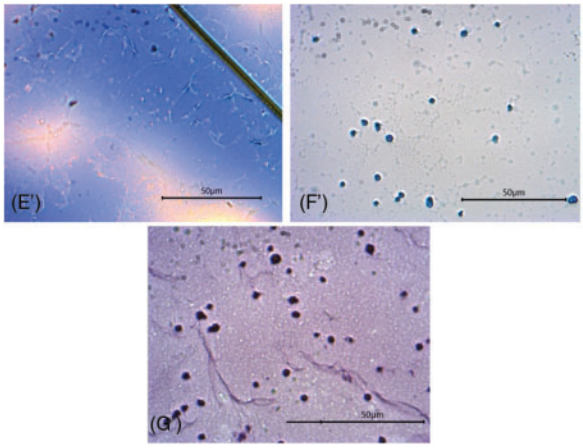


Fig. 6. (E) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr.10 ×, Many platelets, many erythrocytes and many lymphocytes (May-Grûnwald) are highlighted (scale bar 100 μm); (F) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr. 20 ×, Many platelets are highlighted, many erythrocytes (Methylene Blue stain) (scale bar 50 μm); (G) A-PRF liquid 1300 rpm × 5′ (S-PRF sticky green) ingr.40 ×, Many platelets, many erythrocytes and many lymphocytes (May-Grûnwald) are highlighted (scale bar 20 μm); (H) A-PRF liquid 1300 rpm × 5′ (Green S-PRF sticky) input 100 × by immersion; Erythrocytes are highlighted (Methylene Blue stain) (scale bar 10 μm). (E') A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.40 ×, There are few lymphocytes (Methylene Blue colouration) squeezed liquid; (F') A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.40 ×, Many lymphocytes are highlighted (Methylene Blue staining); (G') A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.60 ×, Many platelets are highlighted (May-Grûnwald staining) (in all images scale bar 50 μm).

(17.70%) (Table XI), absence of Monocytes, a reduced quantity of Neutrophilic Granulocytes (2.72%), and Lymphocytes at 46.1% and Fibrinogen at 29.0% compared to the whole blood Table XI, Figs. 5 and 12.

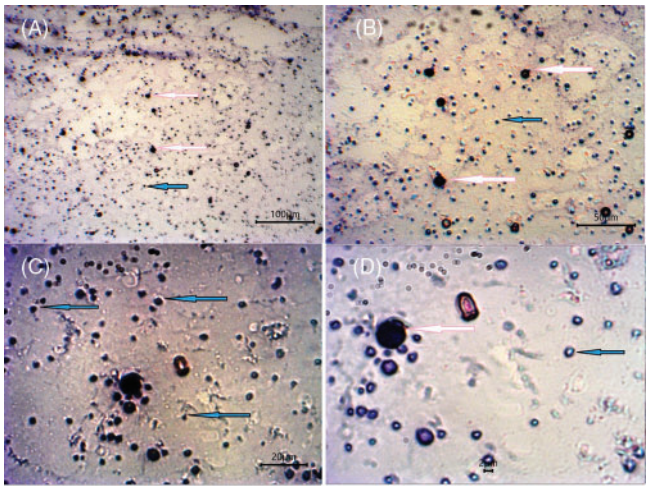
Note that the PRP product does not solidify as the fibrinogen concentration is low.

PRP in the literature appears to be a platelet concentrate with a high to the very high concentration of thrombocytes but with a lower amount of natural fibrinogen [15].

4. Discussion

The efficiency of different protocols in preparing liquid PRF was investigated, but was compared using only one type of centrifuge, and a small number of samples were used for each protocol.

The generation of PRF is a centrifugation-dependent process [16], [17]. Centrifuges work by spinning supernatants around a fixed axis, thereby applying an

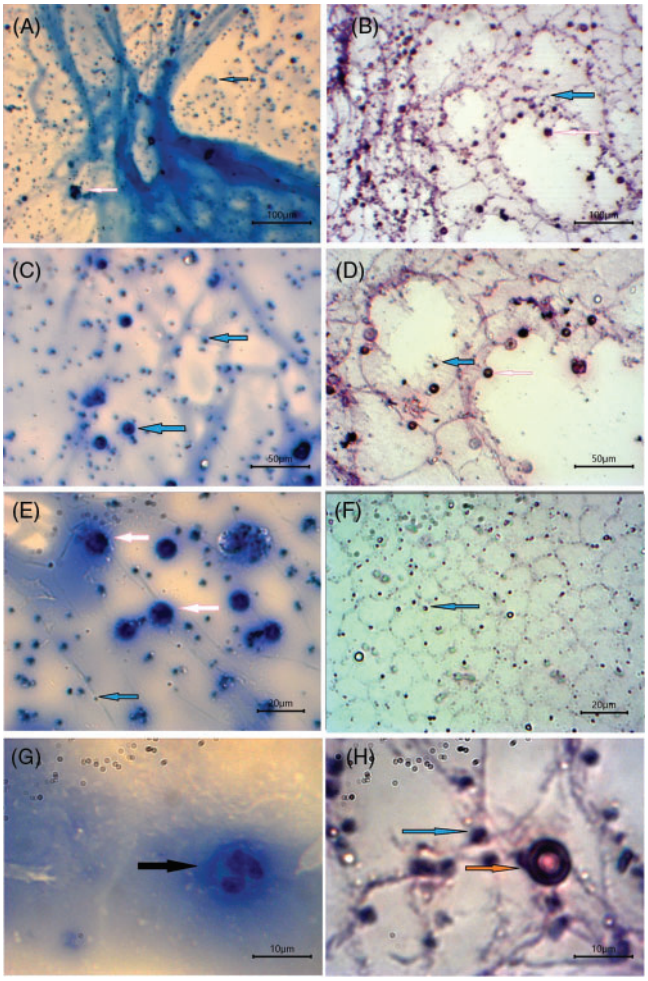


Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 7. Comparison between A-PRF Liquid 1300 rpm × 5′ (Vacumed FL test tube) mixed Toluidine Blue and May-Grûnwald staining. (A) A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.10 x, many platelets and many lymphocytes are highlighted (scale bar 100 µm); (B) A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr. 20 x, Many platelets and many lymphocytes are highlighted (scale bar 50 µm); (C) A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.40 x, Many platelets and lymphocytes are highlighted (scale bar 20 µm); (D) A-PRF liquid 1300 rpm × 5′ (Vacumed FL) ingr.60 x, A lymphocyte and many platelets are highlighted (scale bar 2 µm); in all images mixed Toluidine Blue and May-Grûnwald stains.

acceleration force perpendicular to the axis. Relative centrifugal force (RCF; g-force) is the force of acceleration applied to a sample in a centrifuge, which is directly proportional to the revolutions per minute (RPM) experienced by a sample in a test tube. This resultant force causes various elements in the sample to separate based on the individual weight of its elements and is the basis for blood separation techniques performed by laboratory centrifuges. RCF (g) is measured in multiples of the standard acceleration due to gravity at the Earth's surface and is based on two specific variables including rotor width/radius and rotational speed (RPM). The radius of the centrifuge or rotor is as critical as the RPM in the manufacturing process of a specific RCF. RPM and RCF are related by the formula $RCF = 1.12 \times r \times (RPM/1000)^2$ where r is the center of the distance from the end of the tube to the centrifuge in millimeters. RCF is an important parameter in the production of PRF and must be calculated for each type of centrifuge, especially if this parameter is not present on the machine.

Platelets, as the dominant component of PRF [Injectable Fibrin Scaffold (IFS)], are the major cells responsible for the biological activity of PRF. PRFs contain various platelet-derived protein molecules that are involved in the wound-healing signaling cascade [18]. All these substances are stored by three types of granules (α, δ and λ) located inside the platelets. The IFS, with its loose fibrin network structure, can trap large numbers of white blood cells and platelets, and can continuously produce and secrete growth factors. IFS can promote the migration, proliferation, and

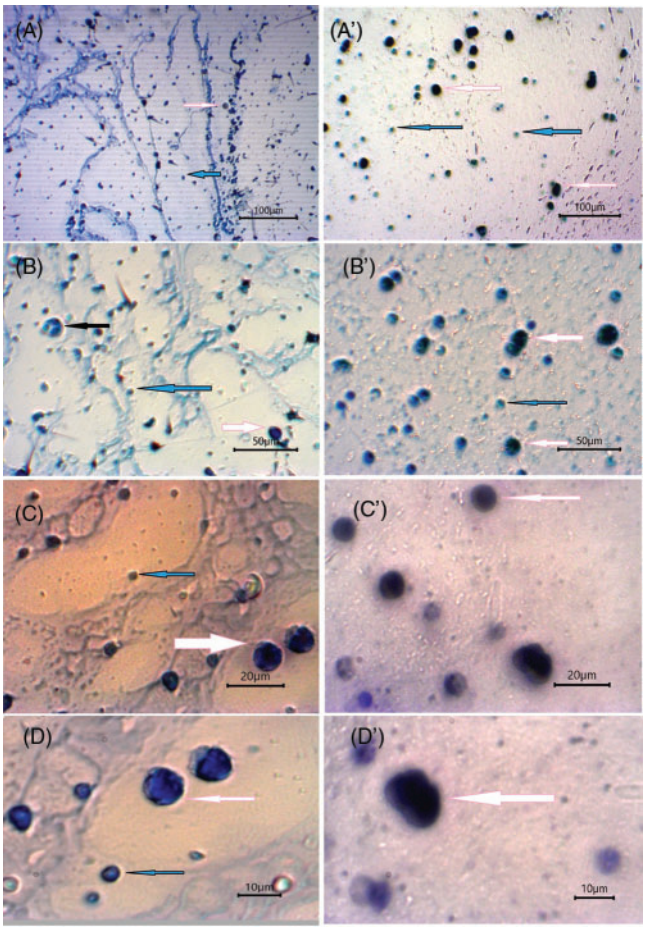


Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 8. Comparison between i-PRF 700 rpm × 5′ (Vacumed FL tube) Methylene Blue staining on the left and May-Grûnwald staining on the right. (A) i-PRF 700 rpm × 5′ (Vacumed FL tube) ingr. 10 x, many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (B) i-PRF 700 rpm × 5′ (Vacumed FL tube) ingr. 10 x, many platelets and many lymphocytes are highlighted (May-Grûnwald) (scale bar 100 µm); (C) i-PRF 700 rpm × 5′ (Vacumed FL) ingr. 20 x, Many Platelets and Neutrophilic Granulocytes (Methylene Blue) are highlighted (scale bar 50 µm); (D) i-PRF 700 rpm × 5′ (Vacumed FL) ingr.20 x, Lymphocytes (May-Grûnwald) (scale bar 50 µm) and many platelets are highlighted; (E) i-PRF 700 rpm × 5′ (Vacumed FL) ingr.40 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 20 µm); (F) i-PRF 700 rpm × 5′ (Vacumed FL) ingr.40 x, Many platelets and few erythrocytes are highlighted (May-Grûnwald stain) (scale bar 20 µm); (G) i-PRF 700 rpm × 5′ (Vacumed FL) ingr.60 x, A Neutrophilic Granulocyte (Methylene Blue) is highlighted (scale bar 10 µm); (H) i-PRF 700 rpm × 5′ (Vacumed FL) input 60 x; An erythrocyte and many platelets are highlighted (May-Grûnwald stain) (scale bar 10 µm).

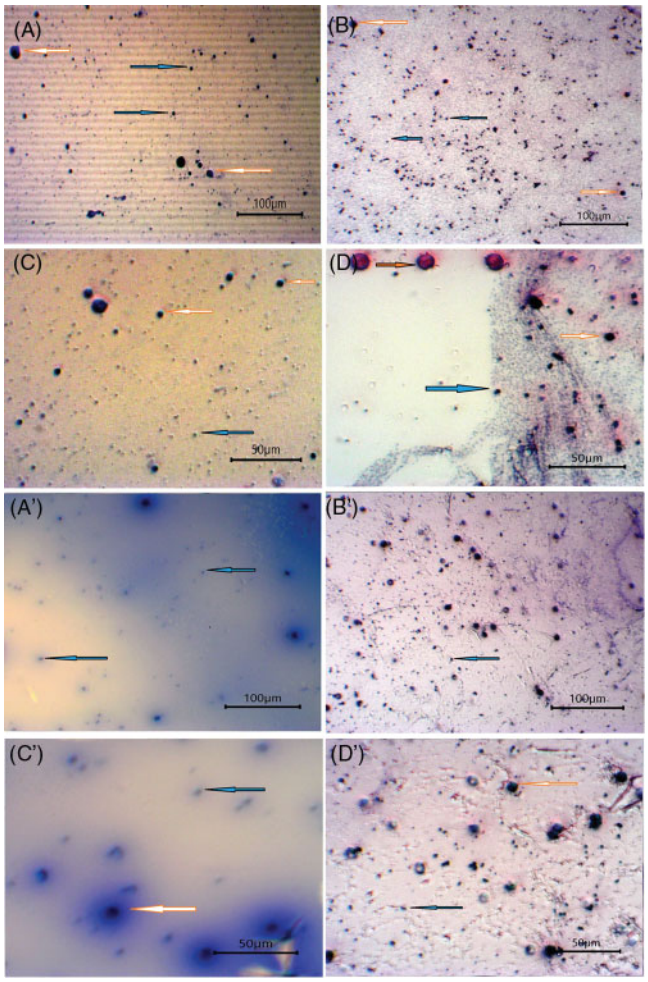
secretion into the matrix of stem cells and skin fibroblasts located around the defect, thanks to its stable and sustained release of growth factors.

Individual clinical situations require different types of application. Therefore, this blood-derived product can be individually adjusted and prepared according to specific



Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 9. Comparison between i-PRF 700 rpm × 5′ (S-PRF sticky green) Methylene Blue staining on the left and May-Grûnwald staining on the right. (A) i-PRF 700 rpm × 5′ (Green S-PRF sticky test tube) ingr. 10 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (A') i-PRF 700 rpm × 5′ (Green tube S-PRF sticky) ingr.10 x, Many platelets and many lymphocytes are highlighted (May-Grûnwald) (scale bar 100 µm); (B) i-PRF 700 rpm × 5′ (S-PRF sticky green) ingr. 20 x, Many Platelets and Lymphocytes and a Neutrophilic Granulocyte (Methylene Blue) are highlighted (scale bar 50 µm); (B') i-PRF 700 rpm × 5′ (S-PRF green sticky) input 20 x, Lymphocytes (May-Grûnwald) (scale bar 50 µm) and very many platelets are highlighted; (C) i-PRF 700 rpm × 5′ (S-PRF sticky green) ingr.40 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 20 µm); (C') i-PRF 700 rpm × 5′ (S-PRF sticky green) ingr.40 x, Many platelets and lymphocytes are highlighted (May-Grûnwald stain) (scale bar 20 µm); (D) i-PRF 700 rpm × 5′ (S-PRF sticky green) ingr. 60 x, Lymphocytes and many platelets are highlighted (Methylene Blue) (scale bar 10 µm); (D') i-PRF 700 rpm × 5′ (Green S-PRF sticky) input 60 x; A lymphocyte and many platelets are highlighted (May-Grûnwald stain) (scale bar 10 µm).

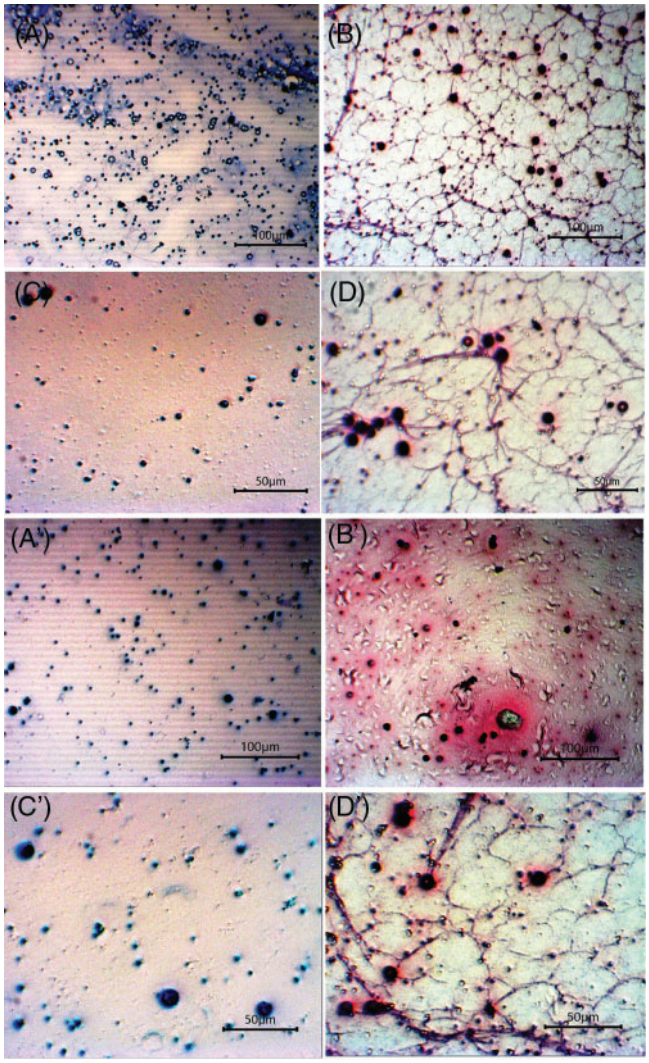


Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 10. Comparison between i-PRF 3300 rpm × 3′ (Vacumed FL and Green S-PRF sticky) Methylene Blue stain on the left and May-Grûnwald stain on the right. (A) i-PRF 3300 rpm × 3′ (Vacumed FL test tube) ingr. 10 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (B) i-PRF 3300 rpm × 3′ (Vacumed FL test tube) ingr.10 x, Many platelets and many lymphocytes are highlighted (May-Grûnwald) (scale bar 100 µm); (C) i-PRF 3300 rpm × 3′ (Vacumed FL) ingr. 20 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 50 µm); (D) i-PRF 3300 rpm × 3′ (Vacumed FL) ingr.20 x, Lymphocytes (May-Grûnwald) (scale bar 50 µm), Erythrocytes and many Platelets are highlighted; (A') i-PRF 3300 rpm × 3 ′ (S-PRF sticky green) ingr.10 x, Many platelets and few lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (B') i-PRF 3300 rpm × 3 ′ (S-PRF green sticky) ingr.10 x, Many platelets and many lymphocytes are highlighted (May-Grûnwald stain) (scale bar 100 µm); (C') i-PRF 3300 rpm × 3 ′ (S-PRF sticky green) input 20 x, Lymphocytes and many platelets are highlighted (Methylene Blue) (scale bar 50 µm); (D') i-PRF 3300 rpm × 3 ′ (Green S-PRF sticky) input 20 x; Many lymphocytes and many platelets are highlighted (May-Grûnwald stain) (scale bar 50 µm).

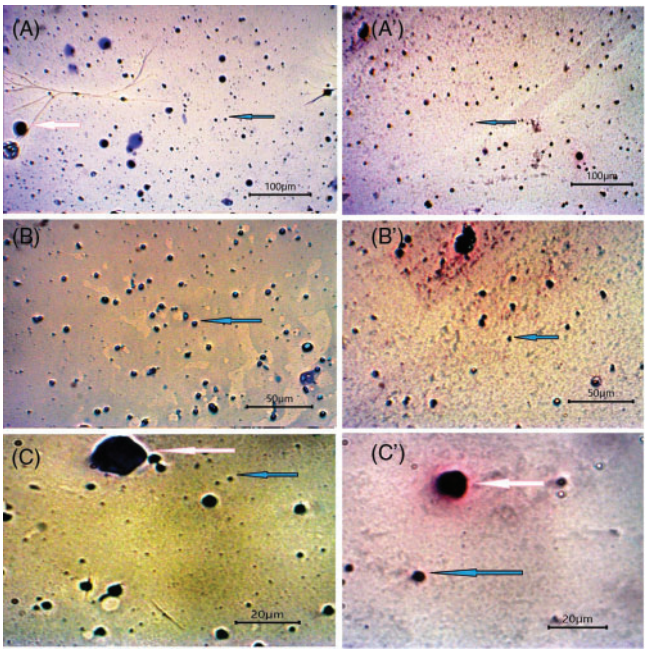
clinical requirements as liquid and solid PRF matrices. Liquid PRF can be used as biologized biomaterials, such as bone substitutes and xenogeneic collagen membranes or even infiltrated into joints or subcutaneous tissue.

As this study was started as an unfunded pilot, it was limited to a small number of participants. To minimize the risk of gender bias in this small number of volunteers, subjects of both sexes with and without peripheral vascular



Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 11. Comparison between Liquid Fibrinogen 2700 rpm × 3′ (Vacumed FL and Green S-PRF sticky). Methylene blue staining on the left and May-Grûnwald on the right. (A) Liquid fibrinogen 2700 rpm × 3′ (Vacumed FL test tube) ingr.10 x, Many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (B) Liquid fibrinogen 2700 rpm × 3′ (Vacumed FL test tube) ingr.10 x, Many platelets and many lymphocytes with fibrin filaments (May-Grûnwald) are highlighted (scale bar 100 µm); (C) Liquid Fibrinogen 2700 rpm × 3′ (Vacumed FL) ingr.20 x, Many Platelets and Lymphocytes are highlighted (Methylene Blue) (scale bar 50 µm); (D) Liquid Fibrinogen 2700 rpm × 3′ (Vacumed FL) ingr.20 x, Lymphocytes (May-Grûnwald) (scale bar 50 µm), Erythrocytes and many Platelets are highlighted; (A′) Liquid fibrinogen 2700 rpm × 3′ (S-PRF sticky green) ingr.10 x, Many platelets and lymphocytes are highlighted (Methylene Blue) (scale bar 100 µm); (B′) Liquid fibrinogen 2700 rpm × 3' (S-PRF green sticky) ingr.10 x, Many platelets and many lymphocytes are highlighted (May-Grûnwald stain) (scale bar 100 µm); (C′) Liquid fibrinogen 2700 rpm × 3′ (S-PRF green sticky) ingr.20 x, Lymphocytes and many platelets are highlighted (Methylene Blue) (scale bar 50 µm); (D′) Liquid Fibrinogen 2700 rpm × 3′ (Green S-PRF sticky) ingr.20 x; Many lymphocytes and many platelets with fibrin filaments are highlighted (May-Grûnwald stain) (scale bar 50 µm).



Legend:
Granulocytes Neutrophils
Red Blood Cells
Platelets
Monocytes
Lymphocytes

Fig. 12. PRP 2200 × 20′ (PRP BioReb Gel); A, B, C Methylene Blue staining; A', B', C' May-Grûnwald stain. A, A' Ingr.10 × (scale bar 100 µm); B, B' Ingr.20 × (scale bar 50 µm); C, C' Ingr. 40× (scale bar 10 µm).

disease were included in this first pilot study. 9 mL of whole blood was centrifuged in plastic tubes (i-PRF S-PRF sticky tubes, Process per PRF™, Nice, France) to obtain PRF Liquid and Vacumed FL PET tubes. The centrifuge (Duo centrifuge, Process for PRF™, Nice, France) used had a fixed angle, no brake, and a rotor size of 110 mm according to the protocol (43.1° rotor angle, 75 mm radius at the center of the pipe, 100 mm maximum and 35 mm minimum).

The importance of determining the amount of fibrinogen present in each fraction and the yield of coagulable components present serves to predict the ability of liquid PRF to generate PRF membranes. Many factors such as fibrinogen can be released from the α granules of platelets to form a clot [4]. The incorporation of fibrinogen into the α-granules occurs through an endocytosis mechanism mediated by the α IIb β3 integrin receptor [19]. The conversion of fibrinogen to fibrin is mediated by thrombin which cleaves fibrinopeptides A and B from the Aα and Bβ, chains, respectively, forming a fibrin monomer which then polymerizes to form a branched network of fibers.

An average platelet accumulation of nearly 1.5-fold was found in liquid fibrinogen compared to whole blood samples. These findings have important significance since higher platelet harvest with higher plasma volume has greater clinical value whereas higher platelet concentration alone has no significance [20], [21].

This concept led us to use a test tube thermostat which in many cases allowed us to obtain a greater quantity of liquid PRF (>3.5 ml).

The L-PRF membrane and the various types of liquid PRF, including liquid fibrinogen, have a high concentration of leukocytes and platelets [22]. The combination of activated platelets in the PRF and fibrinogen results in the mass production of fibrin. More than 80% of the platelets and 72% of the leukocytes of the initial blood sample are present in the PRF. The same is true for liquid fibrinogen with 88% and 70%, respectively. The exudate showed a low cellular content with 2.5% platelets and 0.9% leukocytes [22]–[25].

In this preliminary study, we have shown that the type of Liquid PRF with the highest content of Platelets, Monocytes, Lymphocytes, and Neutrophilic Granulocytes, with a sufficient content of Fibrinogen, is i-PRF (700 rpm × 5′). In particular, the content of Platelets, Monocytes, and Neutrophilic Granulocytes in the i-PRF (700 rpm × 5′) obtained with the Vacumed FL test tube (code 44909) was notable with statistically insignificant differences compared to whole blood (Fig. 5), while the content of Lymphocytes and Fibrinogen obtained with i-PRF (700 rpm × 5′) extracted with PRF-S-Sticky tube is higher.

In comparison, the content of cells and fibrinogen obtained with the two methods of preparation of PRP (Fig. 5E) is much lower, contrary to what was claimed by Rattanasuwan [4] and Pietruszka [9].

5. Conclusions

Our study sought to standardize the liquid PRF preparation procedure, which, while remaining an easy-to-perform and low-cost technique, does not require specialized equipment and has a certain consistency in production in terms of macroscopic, microscopic, and cytological characteristics.

To the authors' knowledge, no study to date has investigated the cellular and fibrinogen content by comparing them in the various types of liquid PRF produced to date with the use of a fixed-angle Duo centrifuge (PROCESS, France). In summary, an injectable fibrin scaffold (IFS) was extracted by one-step centrifugation. This method is relatively simple to apply and produces an easy-to-use platelet concentrate in a liquid formulation. The liquid scaffold contains white blood cells and platelets which can support the release of growth factors. Thus, IFS can be used as a therapeutic agent alone or in combination with other biomaterials to promote tissue regeneration. In fixed-angle devices, the cells are pushed against the wall of the tube and in this process, the larger red blood cells trap the platelets and drag them into the red zone. In horizontal centrifuges there is no such phenomenon; therefore, there is a clear separation of the cells based on their mass so that, in future studies, we want to compare the cellular content with that of the oscillating centrifuge.

We also plan, in the next clinical studies, to use the Liquid PRF that we have evaluated to contain the highest concentration of Platelets and Monocytes (i-PRF 700 rpm × 5′ produced with Vacumed FL Test Tube) (code 44909) (126.27% and 127.59%) to inject it at the edges of an ulcerated wound in the amount of 1 ml every 2 cm subcutaneously to stimulate tissue regeneration and neoangiogenesis [26]. However, we cannot overlook the fact that when obtained from an autologous blood sample, the liquid PRF produced is scarce (~3–3.5 mL per 9 mL of blood drawn) and can only be used in a limited volume.

Author Contributions

Conceptualization: Crisci Michela and Crisci Alessandro; Methodology: Flagiello Fabiana; Formal analysis: Lepore Giovanni and Feleppa Federica; Writing—Preparation of original draft: Crisci Alessandro.

Conflicts of Interest

Authors declare that they do not have any conflict of interest.

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