Cytomorphological analysis of liquid platelet-rich fibrin produced with the DUO fixed angle centrifuge (Process, France) for use in the regenerative therapy of skin ulcers

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ABSTRACT

Liquid platelet-rich fibrin (PRF) is an injectable second-generation platelet concentrate rich in platelets, leukocytes and fibrinogen obtained by centrifugation of autologous blood. The aim of this study is 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 liquid fibrinogen (LF) 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. In this preliminary study we highlighted that the type of liquid PRF has 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 neutrophil granulocytes (64.6% *vs*. 64.8%) and fibrinogen (67.9% *vs*. 87.3%), is the i-PRF (700 rpm×5 min) obtained with Vacumed LF 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 platelet-rich plasma is much lower. This indicates that the liquid PRF is most suitable to be used in various cases of tissue regeneration such as facial aesthetics, intra-articular injection, periulcerative injection, *etc*. is the i-PRF (700 rpm×5 min) obtained with a Vacumed LF tube.

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Introduction

A liquid formulation of platelet-rich fibrin (PRF) can be used alone or in combination with various biomaterials. A liquid formulation of platelet-rich fibrin, called injectable-PRF (i-PRF), was studied by Shao *et al*. by centrifugation at 700 rpm for 3 min (60 g) without anticoagulants.[1] Data suggest that i-PRF can accelerate fibroblast proliferation. Meanwhile, the same authors obtained an injectable fibrin scaffold (IFS) rich in growth factors by centrifugation at 3000 rpm for 10 min. 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*.[2]

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Furthermore, the fibrin network spread in the liquid scaffold promotes the stable and prolonged release of growth factors for more than two weeks (up to 28 days),[3] as well as transporting stem cells. The platelets contained in it regulate the recruitment of adult stem cells towards damaged cells; this may therefore be an important mechanism in regenerative cellular responses.[4] PRF establishes a local environment conducive to MSC migration and can also serve as a source of stem cells.[5] Therefore, this IFS can be used in regenerative medicine alone or as an adjuvant to other biomaterials.

The concentrated platelets present in the i-PRF are responsible for the active secretion of growth factors and the induction of the requirement, proliferation and differentiation of various cells involved in the regeneration process.[4]

Platelet activation begins immediately upon contact with the wall of the centrifugation tube and leads to the formation of a dense fibrin network and a usable PRF clot. The slow polymerization of fibrin allows for greater entrapment of circulating (intrinsic) cytokines in the fibrin matrix. A recent study demonstrated that reducing relative centrifugal force leads to a significant increase in the total number of platelets and leukocytes and the amount of growth factors, indicating that the concept of low-speed centrifugation results in an increase in the regeneration potential of the PRF.[6]

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: i) what concentration of thrombocytes and leukocytes could be collected within the supernatant in different liquid PRF production protocols?; ii) which protocol gives better results in terms of platelets and fibrinogen collected?

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

Materials and Methods

The aim of this study is to discover and confirm the best tube type and 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 liquid PRFs [concentrated-PRF (C-PRF) liquid, advanced-PRF (A-PRF) liquid, and i-PRF], using a 41.3°C fixed angle centrifuge and with different types of 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 network. A comparison was also carried out with the Platelet Rich Plasma (PRP) produced at 2200 rpm×20 min with PRP BioReb Gel (Hettich EBA 200 centrifuge 33°C angle) with single centrifugation and double centrifugation according to the procedure recommended by Rattanasuwan *et al*.[7]

Centrifugation of the PRF in the negative pressure vacuum collection tube in Plastic (PET) Vacumed liquid fibrinogen (LF) (code 44909) and with the original S-PRF Sticky tube (recommended by the manufacturing company Process, France) allows you to prepare the PRF in liquid form and to use it in an injectable manner. Liquid PRF can be used in various cases of tissue regeneration such as facial aesthetics, intra-articular injection, periulcerative injection, *etc*.

C-PRF liquid (RCFclot=525 g; RCFmax=700 g; RCFmin=280 g) sec. Miron 8,9 or other types of liquid PRF are found 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 (neutrophilic granulocytes, monocytes, lymphocytes, and platelets) was evaluated both with a blood count and with a 10-100× Enlargement Optical Microscope with staining. Methylene blue and May-Grůnwald stains were performed to stain the liquid PRF smears to confirm the cellular content determined by hemocytometric examination.

Some researchers worry about a possible health hazard when glass vacuum blood collection tubes with silica activators are used. O'Connell, in fact, described the inevitable contact with silica. For this reason, we only used silica-free tubes.[10]

The study was approved by the local ethics committee and each patient was included in the study after obtaining written consent and then informed about the procedures.

The hematological samples of 45 apparently well-being subjects aged between 35 and 101 years were examined, of which 23 were males and 22 were females.

Inclusion criteria

No use of anticoagulants or equivalent functional medications for one month prior to enrollment and platelet count in the range of 150,000 to 450,000/mL and clotting index determined to be normal [prothrombin time between 11 and 16 seconds]. Hemoglobin concentration >9 mg/dl, serum protein concentration >6 grams/dl, and serum albumin >3 grams/dl.

Absolute contraindications for PRF include 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, patients with low hemoglobin (<10g/mL) or platelet count

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($<1.2\times10^5$/mL). Furthermore, patients taking non-steroidal anti-inflammatory drugs, prednisolone >20 mg/day, and anticoagulant therapy were avoided.

Blood chemistry procedures

Liquid PRF is a second-generation platelet concentrate, which includes various growth factors, thrombocytes, leukocytes, CD34+ stem cells, and fibrinous matrix.[11]

A quantity of 1.5 ml of liquid PRF was placed in a tube with EDTA K3E 5.4 mg to perform a blood count and, using a HECO 5 hematological analyzer (Seac Radim Company), and the concentration of Monocytes and Granulocytes were evaluated neutrophils, lymphocytes and platelets (the average values obtained for each type of liquid PRF produced have been reported).

Fibrinogen was measured by immunoturbidimetric assay with ACL 3000 (Beckman Instrumentation Laboratory).

We, therefore, wanted to evaluate the content of fibrinogen, platelets, monocytes, neutrophil granulocytes, lymphocytes, in the peripheral blood and, again through the execution of a Haemochromocytometric test, in the liquid PRF of various types [A-PRF liquid (1300 rpm×5 min); i-PRF (700 rpm×5 min); i-PRF (3300×3 min); C-PRF (2500 rpm sticky) heated in an incubator at 37°C (Figure 1) to simulate body temperature as much as possible.

We also provided for the production of PRP produced at 2200 rpm×20 min with PRP BioReb Gel Kit (C.O. Biotechology S.r.l., Na, Italy) according to the manufacturer's indications on 25 patients and also for the evaluation of monocytes, neutrophil granulocytes, lymphocytes, platelets with blood count and fibrinogen analysis.[7,12] PRP was also prepared as described by Rattanasuwan *et al*.[7] with minor modifications. Briefly, blood was centrifuged in a cell separator (Hettich EBA 200) at 1,300 revolutions/min (rpm) for 10 min after placing it in a PRP BioReb Gel 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 collected and centrifuged again at 2000 rpm for 10 min in a Vacumed LF tube. The result of the second centrifugation consisted of an upper portion of light yellow supernatant serum and a lower portion tinged with red. The top portion of the serum was discarded and approximately 1.5 mL of concentrated serum and platelets remained in the tube. According to Rattanasuwan *et al*., the resulting PRP should have an increase of 331% compared to the basal concentration of platelets and in any case not less than 200%.[7,12]

Figure 1. Incubator used to maintain the tubes at a constant temperature of 37°C.

Cytological procedures for optical microscopy

PRF-liquid staining shows scattered fibrous reticular structures and white blood cells and platelets are present (Figure 2). In fact, smears of liquid PRF were prepared on microscopy slides and stained with Methylene Blue (30'), May-Grŭnwald (3') or Toluidine Blue (30').

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

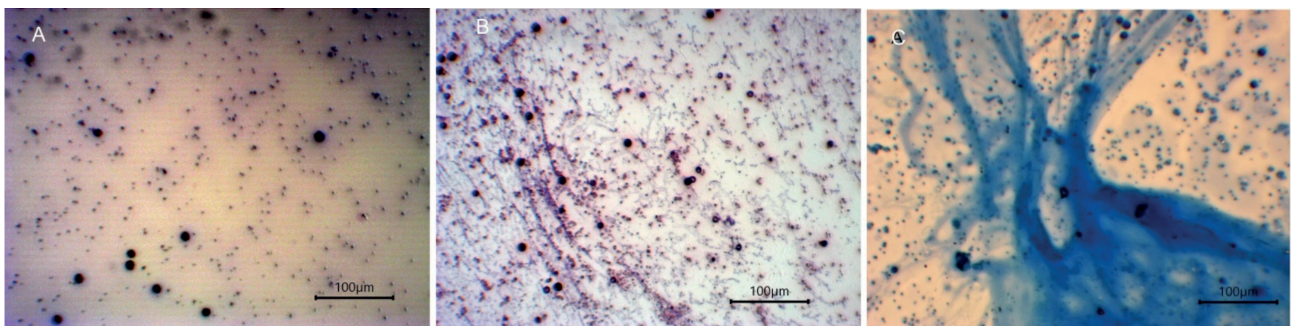

Figure 2. Injectable-platelet-rich fibrin 700 rpm × 5' with Toluidine Blue, May-Grŭnwald and Methylene Blue staining respectively. Many platelets and many leukocytes (lymphocytes) are evident. Ingr.10 × (scale bar 100 microns).

Histomorphometric analysis

The 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 with an original magnification of 10, 20, 40, 60, 100×immersion (Optika, B-150D-BRPL, Optika S.r.l. Italia). The digital images were saved on a computer. AmScope MD 500-CK 5.0 mp software (United Scope, LLC, NNL), Vers.2022 was used for histomorphometric analysis.

Statistical measurements and analyses

The data of the continuous variables obeying the normal distribution were expressed as "Mean ± Standard Deviation" (x ± sd), "Standard Error" and "Median". As regards the statistical significance of the measurement of the cytological and fibrinogen values detected in the PRF-Liquid, for the differences between the groups, it was 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. Any value of P≤0.05 was considered statistically significant, P≤0.005 was highly significant, while P≥0.05 was considered non-significant. Data were analyzed using version 6.0 of the Santon-Glantz 2007 Statistics for Biomedical Disciplines package.

Results

The procedure was well tolerated in all subjects examined. The mean age was 65.07±14.24 years, SE: 2.12; median 66 years.

No significant differences were detected in the baseline hematological comparison of the examined subjects, who had a mean RBC concentration of 4.6×106/mL (±1.1; CI 95%) (P=0.34) (range:4-5.5×106/ mL), WBC of 5.1×103/mL (±0.37; CI 95%) (P=0.24) (range:4.5-8.5×103/mL) and a mean platelet count of 296.8×103/mL (±15.3; CI 95%) (P=0.15) (range:150-400×103/mL). The complete blood count performed on the various types of PRF-Liquid generally shows that IFS includes 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 monocytes. The quantity of liquid PRF obtained from all PRF-Liquids, both with S-PRF Sticky and Vacumed LF tubes, is approximately 2-3 ml. We also tried to heat the tubes used for centrifugation before sampling and, afterward, to more easily obtain PRF-liquid and also a clot from it, using an incubator at 37°C (Figure 1) and favor in this way the formation of fibrin clot by polymerization from fibrinogen under the influence of physiologically available thrombin.

Growth factors epidermal growth factor and vasoendothelial growth factor

Zwittnig *et al*. evaluated growth factors [of particular interest to us are the epidermal growth factor (EGF) and the vasoendothelial growth factor (VEGF)] with a centrifugation process lasting 8 min, with 1200 rpm and a relative centrifugal force (RCF) of 177 g. The produced liquid and solid PRFs were used to analyze the release of five growth factors for 10 days.[13]

Particularly interesting for our studies was the evolution of the release of EGF and VEGF in the comparison between solid and liquid PRF shown in *Supplementary Figure 1*.

After 1 hour, an EGF concentration of 71 pg/mL was measured in the PRF-Liquid. The highest value of 218 pg/mL was highlighted after 7 h. After a sharp 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 it was consistently around 90-100 pg/mL for 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 (*Supplementary Figure 1*).

Solid PRF released 39 pg/mL of EGF after 1 hour but increased to a plateau of 203 pg/mL from hour 7 to day 7. On day 10, it decreased to 40 pg/mL. VEGF values started at 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 (*Supplementary Figure 1*).

Cytological study in optical microscopy

Both C-PRF liquid 2500 rpm×8 min obtained in a Vacumed LF tube and that in S-PRF Sticky tube (RCFcoagulo=525 g; RCFmax=700 g; RCFmin=280 g) had a cellular presence highlighted with Methylene Blue staining ed May-Grŭnwald in low power field (10×, 20× enlargement) [*Supplementary Figure 2* (A, B, E, F) and (C, D, G, H) respectively]. A conformed fibrin network structure was not observed in any of the specimens examined. Blood-derived cellular elements present in various concentrations were identified depending on the centrifuge tube used (*Supplementary Figure 2*).

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 LF tube compared to those with original S-PRF Sticky (*Supplementary Tables 1, 2*) 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 *Supplementary Figure 2* and in the graphs contained in Figure 3.

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

In particular, mononuclear cells (monocytes and lymphocytes): i) modulate the inflammatory process through the local production of a large number of cytokines and other specific substances which initiate the tissue healing process; ii) 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; iii) stimulate local stem cells and progenitor cells to promote reparative processes.[14]

Chronic low-grade inflammation within the vascular wall has been shown to be associated with monocyte infiltration. The maturation of monocytes into macrophages is accompanied by the production of cytokines and growth

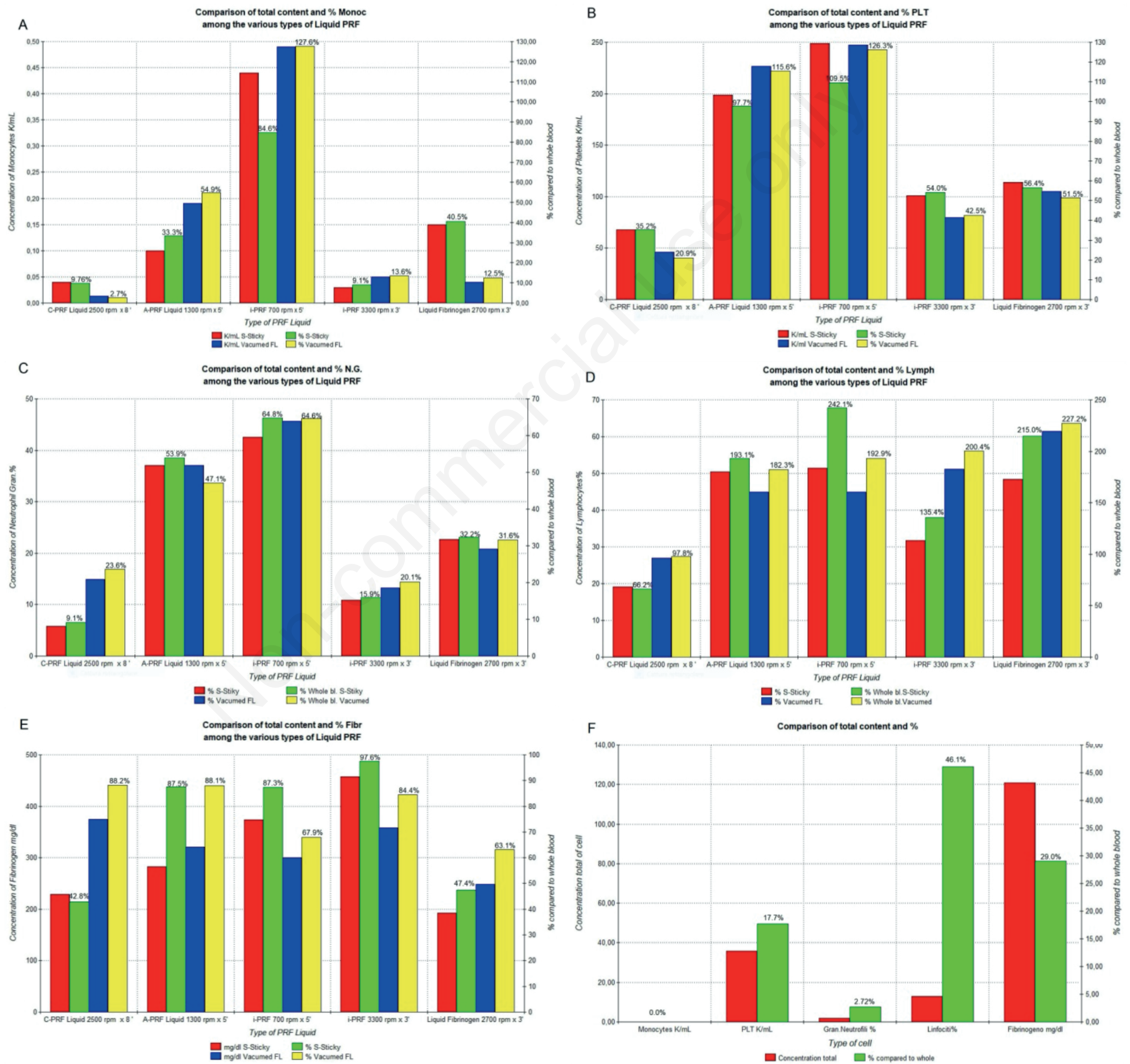

Figure 3. Graphical comparison of the content of monocytes (A), platelets (B), neutrophil granulocytes (C), lymphocytes (D) and fibrinogen (E) in the various types of liquid platelet-rich fibrin produced with original S-PRF Sticky tube and Vacumed Liquid Fibrinogel in absolute values (mg/dl) and in relative percentages in relation to the initial content in whole blood. In F the cellular content is found in platelet-rich fibrin.

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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.[15]

Even with the use of the Vacumed LF tube and with a mixed Toluidine Blue/May-Grŭnwald stain (*Supplementary Figure 3*), a shaped fibrin network was not observed, while blood-derived cellular elements present in various concentrations were identified.

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 LF tube in comparison with those with original S-PRF Sticky tubes (*Supplementary Tables 3, 4*) while the lymphocytes (193.10% *vs*. 182.32%) are higher with the use of S-PRF Sticky tubes and are reported extensively in the captions of *Supplementary Figure 4*, while those with the Vacumed LF tube are shown in the captions of *Supplementary Figure 3* and in the graphs in Figure 3.

In the i-PRF 700 rpm Methylene Blue and May-Grŭnwald staining in the low power field (10×, 20×, 40× and 60× enlargements) and shown in *Supplementary Figure 5* (A, C, E, G), (B, D, F, H) respectively, with the use of a Vacumed LF tube. Again, a well-formed fibrin network structure was not observed in any of the samples examined, except for some thin filaments.

Also with the use of the S-PRF Sticky tube with Methylene Blue and May-Grŭnwald staining [*Supplementary Figure 6* (A, C, E, G), (B, D, F, H) respectively] (engr. 10, 20, 40, 60×) a shaped fibrin network was not observed, while blood-derived cellular elements present in different concentrations reported in *Supplementary Table 5* were identified.

Higher concentrations of platelets (126.27% *vs*. 109.49%), monocytes (127.59% *vs*. 84.62%) compared to whole blood were found in i-PRF liquid obtained with Vacumed LF tube compared to those with original S-PRF Sticky (*Supplementary Tables 5, 6*) 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 *Supplementary Figure 6*, while those with the Vacumed LF are illustrated in the captions of *Supplementary Figure 5* and in the graphs in Figure 3.

The concentration of monocytes present in i-PRF 700 rpm×5' was particularly important for the possible use in vascular regeneration 14 obtained with the Vacumed LF tube (127.59%) compared to the S-PRF Sticky (84.62%). In fact, *in vivo*, monocytes differentiate into endothelial cells and are incorporated into blood vessels.[16]

Also in the i-PRF 3300 rpm the Methylene Blue and May-Grŭnwald staining in the low power field (10×, 20× zoom) in *Supplementary Figure 7* (A, C) and (B, D) respectively, for the Vacumed LF tube. Even in this case, a well-formed fibrin network structure was not observed in any of the examined samples, except for some thin filaments. Even with the use of the S-PRF Sticky tube with the Methylene Blue and May-Grŭnwald staining [*Supplementary Figure 7* (A', C'), (B', D') respectively] (engr. 10, 20×) no a fibrin network was observed, not even just a hint of it, while blood-derived cellular elements present in different concentrations were identified and reported in *Supplementary Tables 7, 8*.

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 the Vacumed ones LF (*Supplementary Tables 7, 8*) 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 LF tubes and are reported in specifically in the captions of *Supplementary Figure 7* and in the graphs in Figure 3.

The platelet concentrate defined as liquid fibrinogen 2700 rpm×3 min obtained in the Vacumed LF tube and in the S-PRF Sticky tube was also examined (RCF clot=408 g; RCF max =653 g; RCF min =326 g) and a cellular presence was found highlighted with Methylene Blue and May-Grŭnwald staining in a low power field (10×, 20× enlargement) in *Supplementary Figure 8* (A, C) and (B, D) respectively, for the Vacumed LF tube.[16] Even in this case, a well-formed fibrin network structure was not observed in any of the samples examined, except for some thin filaments highlighted by May-Grŭnwald staining. Also with the use of the S-PRF Sticky tube and with a staining with Methylene Blue and May-Grŭnwald (*Supplementary Figure 8* [A', C'], [B', D'] respectively) (engr.10, 20×) no shaped fibrin network was observed, while blood-derived cellular elements present in different concentrations were identified, which are reported in *Supplementary Tables 9, 10*.

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 the original S-PRF Sticky tube compared to those Vacumed LF (*Supplementary Tables 9, 10*), while fibrinogen (63.1% *vs*. 47.36%) and Lymphocytes (227.19% *vs*. 215.02%) are higher with the use of Vacumed LF tubes and are specifically reported in the captions of *Supplementary Figure 8* and in the following graphs in Figure 3.

In PRP extracted in 21 patients (2200 rpm, 1147 g×20 min) with 15-mL BioReb Base tube (Biodevice & Advanced Materials S.r.l. Napoli, Italy) with separating and anticoagulant gel [anticoagulant citrate dextrose solution, solution A, USP) with HETTICH EBA200 centrifuge at a fixed angle of 33°C), but also in that produced with dou-

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ble centrifugation there was a cellular presence evidenced by Methylene Blue and May-Grůnwald staining in low-power field (ingr. 10×, 20×, 40×) in *Supplementary Figure 9* (A, B, C) and (A', B', C') respectively.[7,17] Again, a well-constituted fibrin network structure was not observed in any of the samples examined. A low concentration of Platelets was found compared to whole blood (11.54%) (*Supplementary Table 11*), absence of monocytes, reduced amount of neutrophil granulocytes (1.99%) and lymphocytes at 28.7% and fibrinogen at 36.9% compared to whole blood (*Supplementary Table 11*, Figure 3, *Supplementary Figure 9*).

Note that the PRP produced does not solidify because the concentration of fibrinogen is low. PRP in the literature appears to be a platelet concentrate with a high to very high thrombocyte concentration, but with a lower amount of natural fibrinogen.[18]

Discussion

The efficiency of different protocols in preparing liquid PRF was analyzed, 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.[18-20] Centrifuges work by rotating supernatants around a fixed axis, thus applying an accelerating force perpendicular to the axis. Relative centrifugal force (RCF; g-force) is the accelerating force applied to a sample in a centrifuge, which is directly proportional to the revolutions per minute (RPM) to which a sample in a test tube is subjected. This resultant force causes the separation of various elements in the sample 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 that include rotor width/radius and rotational speed (RPM). The radius of the centrifuge or rotor is as critical as the RPM in the process of producing 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 between the end of the tube and 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 preset on the machine.

Platelets, as the dominant component of PRF (IFS), represent the main cells responsible for the biological activity of PRF. PRFs contain various platelet-derived protein molecules that are involved in the wound-healing signaling cascade.[21] All these substances are stored by three types of granules (α, δ and λ) located inside the plates. 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 migration, proliferation, and 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 applications. Therefore, this blood-derived product can be individually adjusted and prepared according to specific clinical requirements such as liquid and solid PRF matrices. Liquid PRF can be used to biologize biomaterials, such as bone substitutes and xenogenic collagen membranes or infiltrates in periulcerative sites.

Because this study was initiated as a pilot, with no funding, it was limited to a small number of participants. In order to minimize the risk of bias due to different genders in this low number of volunteers, subjects of both sexes with and without peripheral vascular disease were included in this first pilot study. 9 mL of whole blood was centrifuged in plastic tubes (i-PRF and S-PRF Sticky tubes, process for PRF™, Nice, France) to obtain PRF liquid and Vacumed LF 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 (rotor angle of 43.1°C, radius of 75 mm in 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 the 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.[7] The incorporation of fibrinogen into α-granules occurs through an endocytosis mechanism mediated by the integrin α IIb β3 receptor.[22]

The conversion of fibrinogen into fibrin is mediated by thrombin which cleaves fibrinopeptides A and B from the A α and B β chains respectively, forming a fibrin monomer which then polymerizes to form a branched network of fibres.

An average platelet accumulation of almost 1.5 times was found in liquid fibrinogen compared to whole blood samples. These findings have important significance because a higher platelet yield with a higher plasma volume has greater clinical value and a higher platelet concentration alone is of no significance.[23,24]

This concept led us to use a tube thermostat which allowed us in numerous cases 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.[25] The combination of activated platelets in the PRF and Fibrinogen results in mass production of fibrin. More than 80% of the platelets

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and 72% of the leukocytes of the initial blood sample are present in the PRF. The same goes for liquid Fibrinogen with 88% and 70% respectively. The exudate showed a low cellular content with 2.5% platelets and 0.9% leukocytes.[25-28]

In this preliminary study, we have highlighted that the type of Liquid PRF with the highest content of platelets, monocytes, lymphocytes and neutrophil granulocytes, with a sufficient content of Fibrinogen, is the i-PRF (700 rpm×5 min). In particular, the content of platelets, monocytes and neutrophil granulocytes in the i-PRF (700 rpm×5') obtained with the Vacumed LF tube (code 44909) was notable, with statistically insignificant differences compared to whole blood (Figure 3), while the content of lymphocytes and fibrinogen obtained with i-PRF (700 rpm×5') extracted with a PRF-S-Sticky tube is higher.

In comparison, the content of cells and Fibrinogen obtained with the two PRP preparation methods is much lower (Figure 3E), contrary to what Rattanasuwan and Pietruszka claimed.[7,12]

Conclusions

Our study attempted to standardize the preparation procedure of Liquid PRF, 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 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 that can support the release of growth factors. Therefore, 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 this phenomenon does not exist; therefore, there is a clear separation of the cells based on their mass, so in future studies, we want to compare the cellular content with that of the oscillating centrifuge.

Furthermore, in the next clinical studies, we expect to use the Liquid PRF which we have evaluated to contain the highest concentration of Platelets and Monocytes (i-PRF 700 rpm×5 min produced with Vacumed LF Tube) (code 44909) (126.27% and 127.59%) in order to inject it at the edges of an ulcerated wound in the quantity of 1 ml every 2 cm subcutaneously to stimulate tissue regeneration and neoangiogenesis.[14,29] Autologous cell therapy represents, in fact, an innovative therapy for critical ischemia of non-revascularizable limbs. The main benefits of cell therapy are the induction of therapeutic angiogenesis with the formation of collaterals which lead to increased blood flow in the ischemic limb and tissue regeneration in non-healing trophic skin lesions. Autologous cell therapy is more effective than conventional treatment for non-revascularizable CLI (NO-CLI).[30] Peripheral blood mononuclear cells used in autologous cell therapy are a heterogeneous population composed of CD34- cells, lymphocytes and monocytes, and from CD34+ hematopoietic stem cells and EPCs, although present in low concentrations.[31]

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