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ORIGINAL ARTICLE

A novel method for harvesting concentrated platelet-rich fibrin (C-PRF) with a 10-fold increase in platelet and leukocyte yields

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Abstract

Background and objectives Liquid platelet rich fibrin (PRF; often referred to as injectable PRF) has been utilized as an injectable formulation of PRF that is capable of stimulating tissue regeneration. Our research group recently found that following standard L-PRF protocols (2700 RPM for 12 min), a massive increase in platelets and leukocytes was observed directly within the buffy-coat layer directly above the red blood cell layer. The purpose of this study was to develop a novel harvesting technique to isolate liquid PRF directly from this buffy coat layer and to compare this technique to standard i-PRF.

Materials and methods Standard high g-force L-PRF and low g-force i-PRF protocols were utilized to separate blood layers. Above the red blood corpuscle layer, sequential 100-μL layers of plasma were harvested (12 layers total; i.e., 1.2 mL, which represents the total i-PRF volume), and 3 layers (3 × 100 μL) were harvested from the red blood cell layer to quantify blood cells. Each layer was then sent for complete blood count (CBC) analysis, and the cell numbers were quantified including red blood cells, leukocytes, neutrophils, lymphocytes, monocytes, and platelets. The liquid PRF that was directly collected from the buffy-coat layer following L-PRF protocols was referred to as concentrated PRF (C-PRF).

Results The i-PRF protocol typically yielded a 2- to 3-fold increase in platelets and a l.5-fold increase in leukocyte concentration from the 1- to 1.2-mL plasma layer compared to baseline concentrations in whole blood. While almost no cells were found in the first 4-mL layer of L-PRF, a massive accumulation of platelets and leukocytes was found directly within the buffy coat layer demonstrating extremely high concentrations of cells in this 0.3–0.5-mL layer (~ 20-fold increases). We therefore proposed harvesting this 0.3- to 0.5-mL layer directly above the red blood cell corpuscle layer as liquid C-PRF. In general, i-PRF was able to increase platelet numbers by ~ 250%, whereas a 1200–1700% increase in platelet numbers could easily be achieved by harvesting this 0.3–0.5 mL of C-PRF (total platelet concentrations of > 2000–3000 × 10^9 cells/L).

Conclusion While conventional i-PRF protocols increase platelet yield by 2-3-fold and leukocyte yield by 50%, we convincingly demonstrated the ability to concentrate platelets and leukocytes over 10-fold by harvesting the 0.3–0.5 mL of C-PRF within the buffy coat following L-PRF protocols.

Clinical relevance Previous studies have demonstrated only a slight increase in platelet and leukocyte concentrations in i-PRF. The present study described a novel harvesting technique with over a 10-fold increase in platelets and leukocytes that can be further utilized for tissue regeneration.

Keywords Fibrin · Blood platelets · Wound healing · Platelet-rich fibrin

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Introduction

Platelet concentrates have been utilized in dentistry and medicine for over three decades due to their ability to release supra-physiological doses of autologous growth factors [1, 2]. Platelet-rich plasma (PRP) was first developed for use in regenerative dentistry, but its use is also widespread in maxillofacial surgery, orthopedic surgery, and esthetic medicine due to its ability to rapidly stimulate tissue neoangiogenesis [3–7]. Proper protocols utilizing PRP typically involve the use of anticoagulants and high g-forces to selectively layer blood cells based on density. Due to these high g-forces, a top platelet-poor layer (acellular) is typically produced within the upper layer, followed by a platelet-rich layer (buffy coat; middle layer), and a bottom red blood cell (corpuscle) layer. Since anticoagulants are utilized, cell layers are separated without fear of coagulation, and typical centrifugation cycles typically range from 15 min to 1 h. Despite the success of PRP, various concerns have been raised due to their use of anticoagulants which have been shown to negatively impact tissue regeneration [3, 8, 9].

Platelet-rich fibrin (PRF) was therefore developed with the aim of removing anticoagulants [10]. As a result, the spin cycles are typically much shorter. Following centrifugation, a three-dimensional fibrin matrix is produced that may serve as a tissue engineering scaffold for various medical procedures that necessitate either soft- or hard-tissue regeneration [11–13]. In dentistry alone, PRF has been utilized for the treatment of extraction sockets [14–17], gingival recessions [18–20], palatal wound closure [21–23], the regeneration of periodontal defects [24], and hyperplastic gingival tissues [25]. In other medical fields, PRF has been utilized for the successful management of hard-to-heal leg ulcers, including diabetic foot ulcers, venous leg ulcers, and chronic leg ulcers [26]. The reported advantages include faster healing, increases in angiogenesis, lower costs (when compared to PRP), and complete immune biocompatibility [27–30].

By utilizing even shorter centrifugation cycles and by modifying the centrifugation tubes, our group demonstrated the advantages of an injectable PRF (termed i-PRF) compared to PRP [31]. Furthermore, a number of studies have further demonstrated that the cellular activity of i-PRF is superior to that of PRP [31–34].

Recently, our research group developed a novel technique to quantify cells within platelet concentrates following centrifugation by sequentially pipetting 1-mL layers of blood following centrifugation [35]. This highly effective quantification method reveals the exact concentration/location of various blood cells following centrifugation and allows a direct investigation of PRF protocols based on the final cell composition. Surprisingly, following L-PRF protocols, a large number of cells accumulated within the buffy coat directly above the red blood cell layer (1-mL sample), with very few cells found throughout the upper PRF clot [35]. We therefore aimed to address two specific questions in the current study: (1) What was the total volume above the red blood corpuscle layer and within the buffy coat in which the majority of these cells were located; and (2) what final concentration of platelets and leukocytes could be harvested by selectively collecting the cells found within this "buffy-coat" region when compared to conventional i-PRF protocols.

Materials and methods

Preparation of PRF

Blood samples were collected with the informed consent of six volunteer donors. All procedures performed in this study involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. No internal review board (IRB) was required for this study because the human samples were not identified [31]. The factors that affect fibrin clot formation and structure include genetic factors, acquired factors (such as an abnormal concentration of thrombin and factor XIII in the plasma, blood flow, platelet activation, oxidative stress, hyperglycemia, hyperhomocysteinemia, medications, and cigarette smoking), and other parameters (such as microgravity, pH, temperature, reducing agents, and the concentration of chloride and calcium ions) [36]. All patients with any of the above conditions were excluded. All included patients were systemically healthy, nonsmoking, and not taking any medications.

The following two centrifugation devices were utilized in this study: an IntraSpin Device (IntraLock, Boca Raton, Florida, USA) and a Duo Quattro (Process for PRF, Nice, France). Two separate protocols were tested. On the Intraspin device, an ~ 700 RCF-max (~ 400 RCF-clot) for 12 min was utilized for the L-PRF protocol, whereas an ~ 60 RCF-max for 3 min was utilized for the i-PRF protocol.

Each of the six volunteers donated three blood collection tubes (10-mL plastic tubes) for each of the two test groups, for a total of six tubes per participant. Additionally, one blood sample was collected to quantify control whole blood. Specific plastic hydrophobic tubes (Chixin Biotech, Wuhan, China) were utilized to prevent clotting during centrifugation.

For one participant, an additional two tubes were harvested to demonstrate the general location of cells following centrifugation using the L-PRF and i-PRF protocols by utilizing the previously proposed 1-mL sequential pipetting method [35] (presented in Figs. 1a and 2). Since we previously observed a massive accumulation of cells within the buffy layer in a 1-mL sample range following the L-PRF protocol, we aimed to precisely investigate the volume in which these cells were located

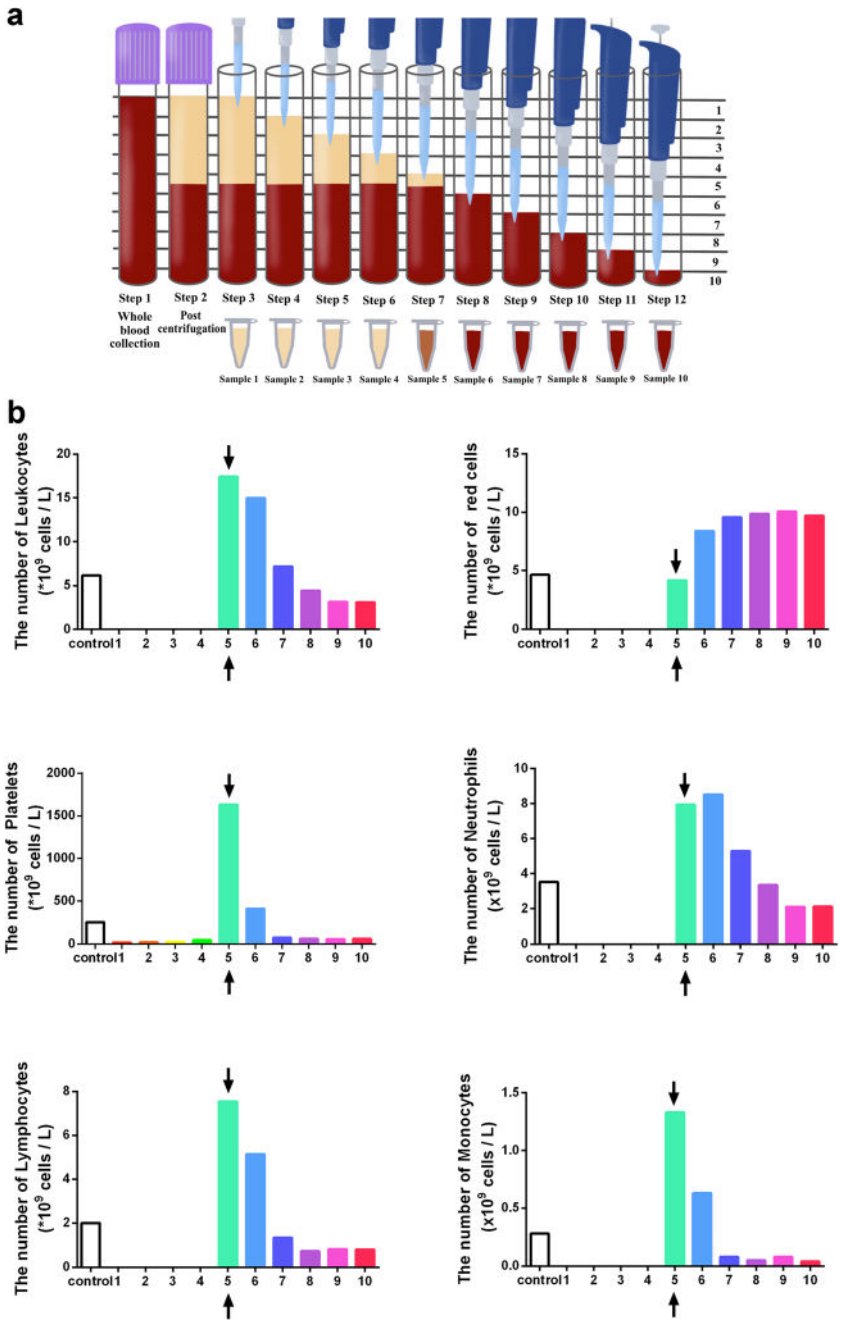

Fig. 1 (**a**) Illustration demonstrating the proposed novel method to quantify cell types following centrifugation. Currently, one limitation reported with standard quantification methods is that whole blood is compared to the total plasma concentration following centrifugation. This, however, does not give a proper representation of the location of cells following centrifugation. By utilizing the proposed technique in this study and sequentially pipetting a 1-mL volume from the top layer moving downwards, it is possible to perform CBC analysis on each of the 10 samples and to accurately determine the precise location of each cell type following centrifugation with various protocols. One layer (in this case, layer 5) will contain some yellow plasma and red blood cells. This is typically the location of the buffy coat, where a higher concentration of platelets/leukocytes is typically located. (**b**) The concentration of the cell types in each layer from 1 mL down to the 10th milliliter sample utilizing the L-PRF protocol (2700 RPM for 12 min; ~ 700 g). The majority of platelets, leukocytes, and monocytes accumulated in the fifth layer of the buffy coat. The first four layers of this plasma layer were typically devoid of all cells. Reprinted with permission [35]

within this 1-mL layer directly above the red cell corpuscle layer. Thus, we developed a novel methodological approach in which 100-μL sequential layers were pipetted starting from ~ 1.2- to 1.5-mL layers above the buffy coat, until reaching the red blood cell layer (Figs. 3a and 4a, depicted as the + 1 to + 12 layer). Additionally, three layers were harvested from the red blood cell layer to determine the number of cells in this layer (Figs. 3a and 4a, depicted as the − 1 to − 3 layer). Each of these 100-μL layers was sent for CBC analysis.

The second tube from each group was utilized to determine the final concentration from the liquid version of the i-PRF yellow plasma layer and compared to those from the average sequential 100-μL layers. For L-PRF protocols, one tube was utilized to harvest 0.5 mL of concentrated PRF (defined as the 0.5-mL buffy coat directly above the red blood corpuscle). This layer is referred to as concentrated PRF (C-PRF) throughout this article because it was harvested from the concentrated buffy-coat layer. Similarly, 0.3 mL of C-PRF liquid was harvested from this layer as well. The blood draw for the 100-μL sequential analysis was carried out with anticoagulants to allow blood samples to be sent for CBC analysis, during which the total number of leukocytes, red blood cells, platelets, neutrophils, lymphocytes, and monocytes was determined in each sample. Thereafter, the data from each sample

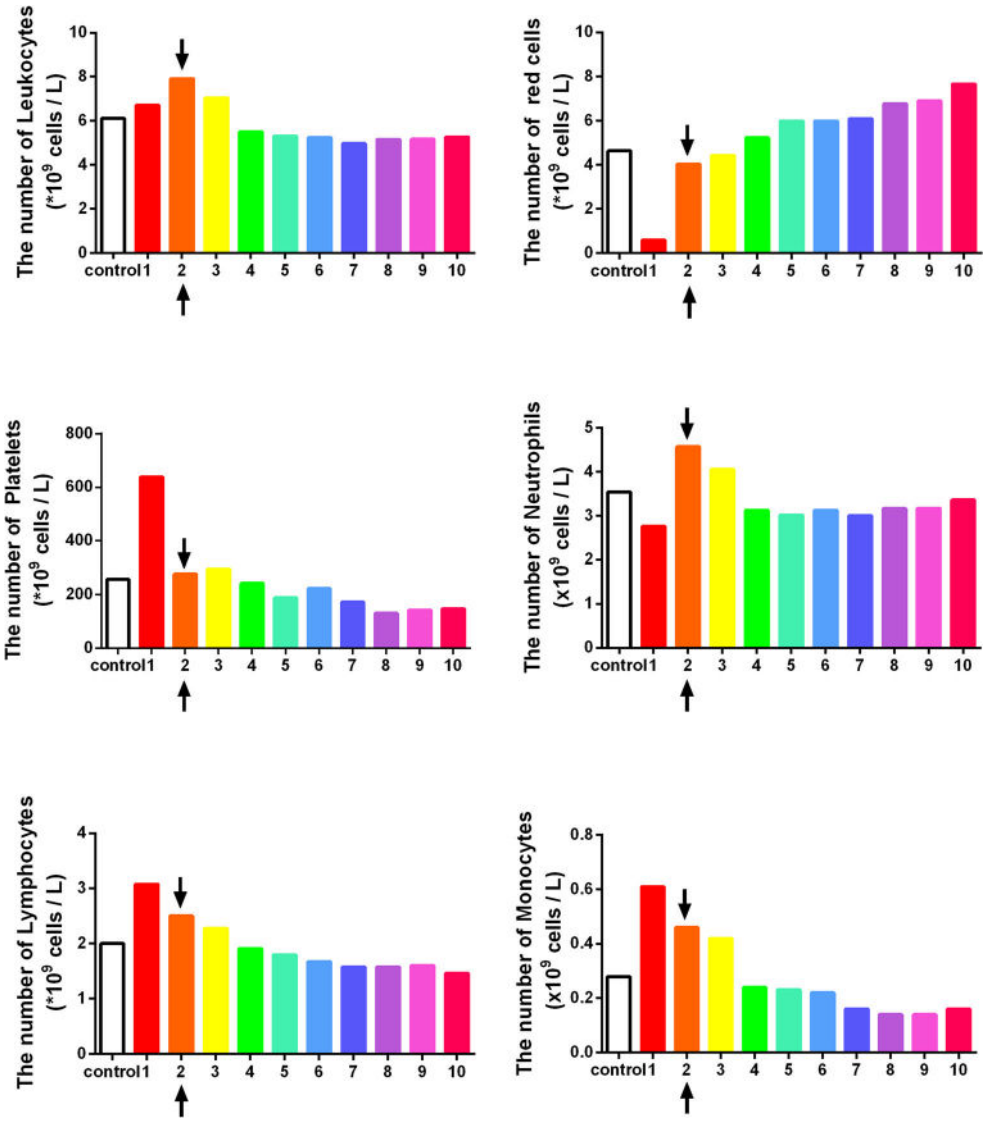

Fig. 2 The concentration of the cell types in each layer from 1 mL down to the 10th milliliter sample from the injectable i-PRF protocol (800 RPM for 3 min; ~ 60 g). There was very little change in the platelet or leukocyte accumulation utilizing this centrifugation protocol. However, a slight increase in platelets and leukocytes was observed in the upper 1–2-mL layers compared to the control

were displayed graphically using GraphPad Prism 8.0 software and were analyzed with one-way ANOVA (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Comparison of cell separation following centrifugation of L-PRF and i-PRF

First, a novel method to investigate cell types following centrifugation utilizing 1-mL sequential pipetting was used to investigate which layers contained the various cell types following centrifugation at both high (L-PRF) and low (i-PRF) g forces (Figs. 1b and 2). Interestingly, in the L-PRF protocol, the majority of platelets and leukocytes (monocytes and lymphocytes) accumulated in layer 5, representing the buffy coat transition between the plasma layer and red blood cell layer. Within this layer, a general 3- to 5-fold increase in the cell concentration was observed compared to that in the control whole blood sample (Fig. 1b). In contrast, the i-PRF protocol demonstrated an average 1.5- to 2.5-fold increase in the various cell types in layers 1–2 (Fig. 2). Since the i-PRF protocol generated a higher concentration of platelets and leukocytes than the L-PRF protocol (because the i-PRF volume was ~ 1–1.3 mL vs the 4.5–5 mL volume in L-PRF), we therefore sought to precisely collect the layer within the buffy coat of the L-PRF protocol (zone 5), where a massive increase in cell numbers was observed directly within the buffy coat.

Sequential pipetting of 100-μL layers in i-PRF and L-PRF

The first question that our group aimed to address was the total volume of the cell-rich liquid above the buffy coat in which platelets and leukocytes were located following the L-PRF

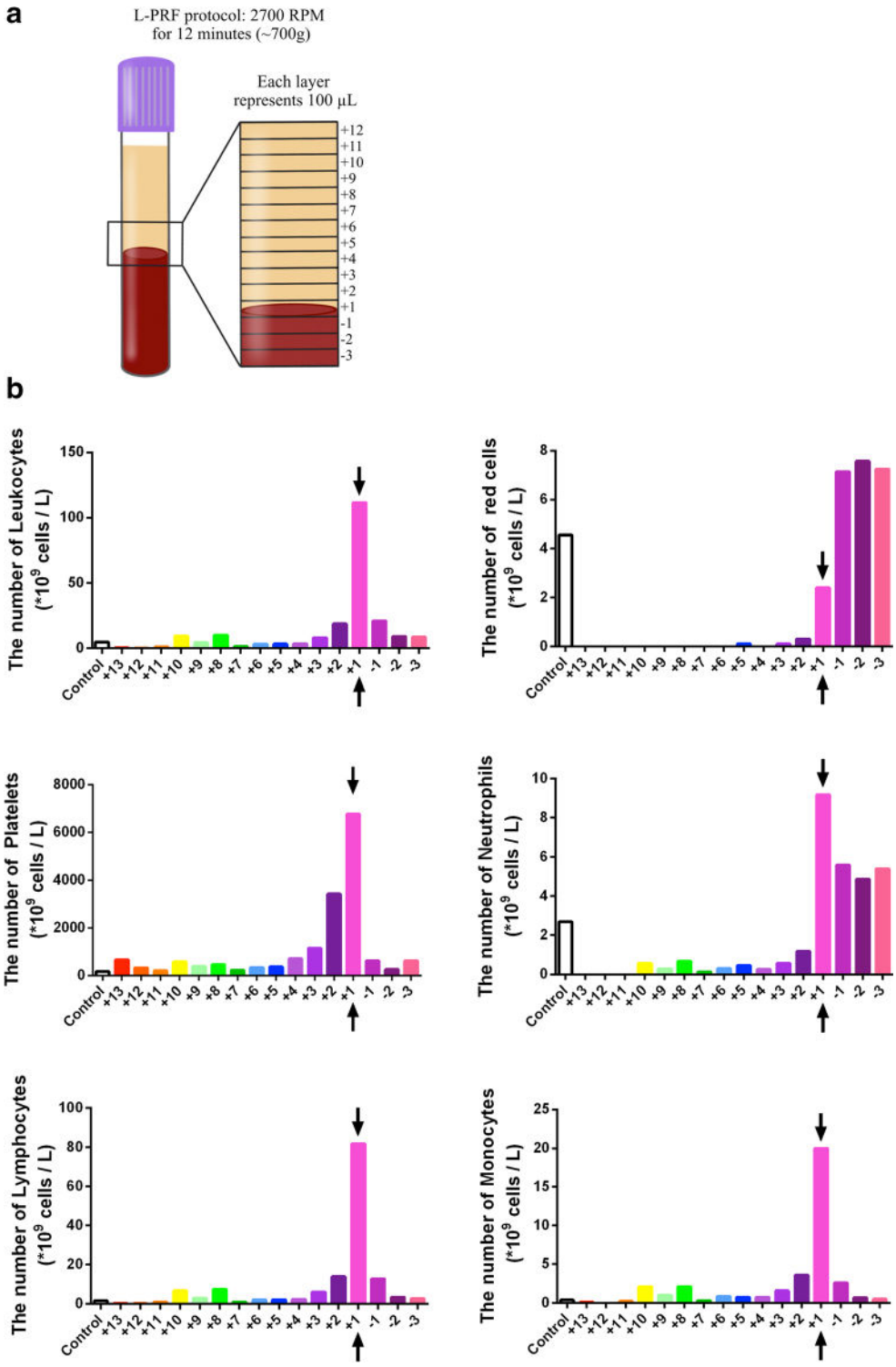

Fig. 3 (**a**) A second methodological illustration depicting the sequential harvesting technique. Briefly, as the majority of cells were found to accumulate within 1 mL of the buffy coat, we sought to precisely investigate the total volume of liquid (mL) above the buffy coat in which cells were concentrated. Following the L-PRF protocol, the upper 3.5 mL was removed, followed by sequential 100-µL layer pipetting and CBC analysis. Three layers of the red blood cell layer were also harvested. **b** The concentration of the cell types in each of the 12 layers (100 µL each) above the red blood cell layer following the L-PRF protocol. There was a massive increase in platelets (roughly a 20-fold increase specifically at the buffy coat layer, between the yellow and red blood cell layers). Interestingly, all cells seemed to accumulate within 3–5 layers (300–500 µL) above the red blood corpuscle

protocol. To address this question, the first 3.5 mL of the upper-plasma layer was removed (acellular layer) from the tube (leaving 1.0–1.5 mL of sample above the red blood cell layer). Thereafter, a sequential pipetting method was once again utilized again from the top down with sequential 100-µL layers to accurately determine how many layers above the red cell layer contained cells (Fig. 3a). Furthermore, 300 µL of the red blood corpuscle layer was also harvested and quantified in 100-µL sequential layers (Fig. 3a). In contrast, the entire i-PRF layer was collected starting from the upper 100-µL layer and was sequentially pipetted until all the plasma layers were collected (Fig. 4a). Once again, 300 µL was

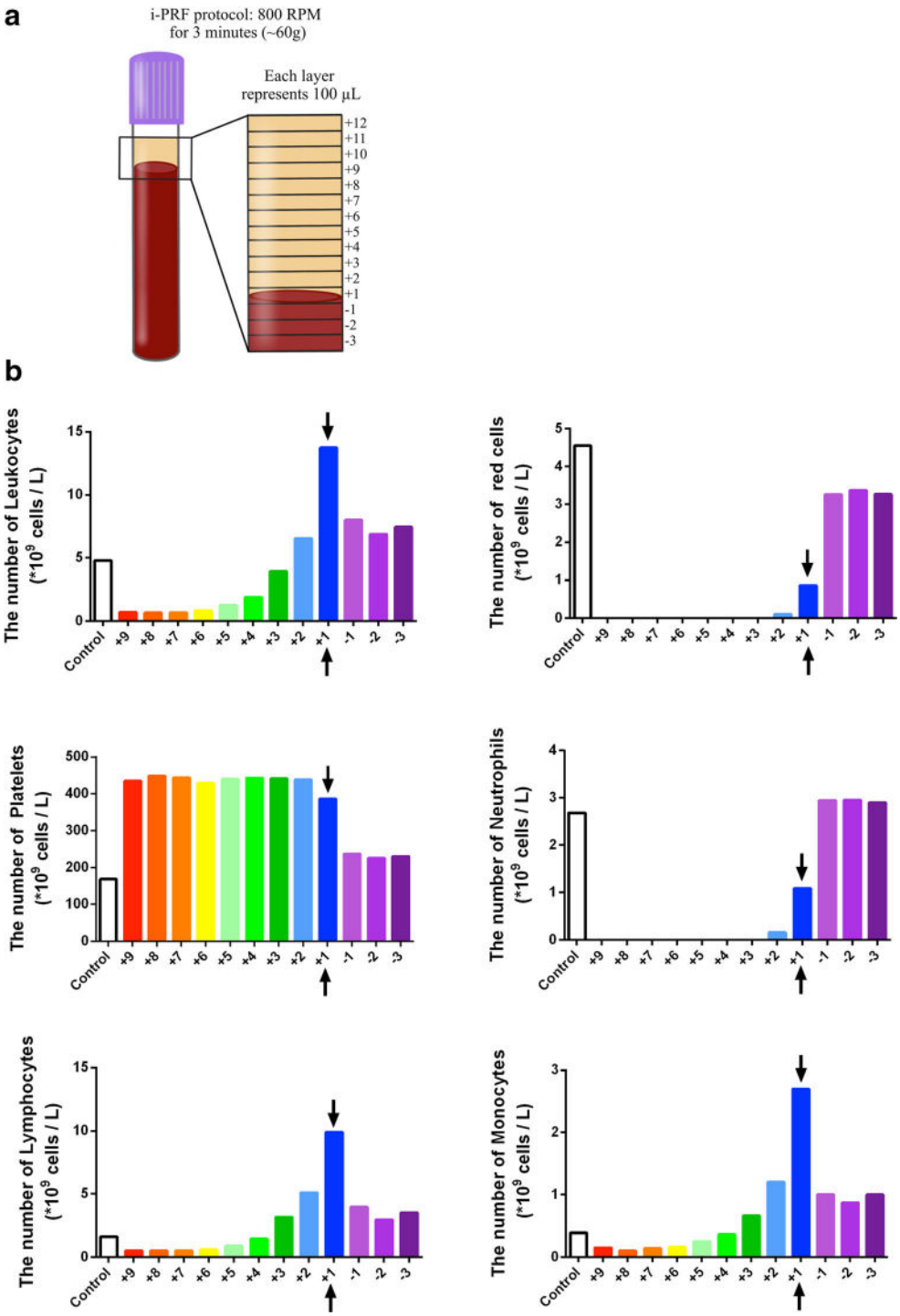

Fig. 4 (**a**) A second methodological illustration depicting the sequential harvesting technique. Similar to L-PRF, all plasma layers of the i-PRF protocol were also harvested in 100-µl sequential layers. Three red blood cell layers (100 µL each) were also collected. (**b**) The concentration of cell types in each layer from 100-µL layers in i-PRF and three layers in the red cell layer. A typical 2–3-fold increase in platelets was observed, whereas only a slight increase in leukocytes and other cell types was observed directly at the separating layer between the plasma and red blood cell layers

sequentially pipetted in 100-µL layers from the red blood corpuscle layer.

Figure 4b demonstrates the results from the sequential 100-µL layers in the i-PRF protocol. In layer 13, there was a 3-fold increase (from 5 to 15 × 10^9 cells/L) in leukocytes directly at buffy-coat layer 13 (represented by arrows). There was also a 5- to 6-fold increase in monocytes. Following the i-PRF protocol, a 2.5-fold increase (from a baseline of ~ 220 to ~ 550 × 10^9 platelets/L) in platelets in all 13 layers (1.3 mL, i.e., thirteen 100-µL samples) was observed, with only a slight increase in leukocyte concentrations.

In contrast, Fig. 3b shows the results from the sequential 100-µL layers above the red blood layer that were found following the L-PRF protocol. Interestingly, almost all the cells accumulated within three layers (i.e., 300 µL) above the red blood corpuscle (Fig. 3b). Most

surprisingly, within this layer, a massive increase in platelets, monocytes, leukocytes, and lymphocytes was observed. For instance, an increase from 225 to 6000 × 10^9 platelets/L was observed, representing a > 25-fold increase in the platelet concentration compared to that at baseline; this specifically occurred within 100 μL above the red blood cell layer.

Based on these results, we assumed that a 0.3- to 0.5-mL layer of concentrated PRF (C-PRF) could be preferentially collected within this buffy coat directly above the red cell layer (Fig. 5a). Figure 5b demonstrates that while the i-PRF protocol increases the leukocyte numbers 1.23-fold, a marked and significant increase in the leukocyte concentration was observed (4.62- and 7.34-fold increases) in both the 0.5-mL and 0.3-mL C-PRF, respectively. Even more strikingly, while i-PRF protocols have typically been shown to increase platelet yields between 200 and 300%, the C-PRF protocols massively increased platelet yields 1138% and 1687%, respectively (Fig. 5c). A similar trend was also observed for monocytes (Fig. 5d). The total after averaging the data from six patients are summarized in Table 1.

Table 1 Properties of blood cells

	Platelets	WBC	RBC
Density (kg/m^3)	1040–1065	1055–1085	1095–1100
Frequency (1/μL)	200,000	5000	5,000,000
Surface (μm^2)	28	330	140
Radius (μm)	11.5	5–7.5	4
Volume (μm^3)	14	200	92
Shape	Irregular disk	Spherical	Biconcave

Discussion

From approximately 2014–2016, the low speed centrifugation concept (also referred to as the LSCC) was believed to isolate the maximum number of cells and the most growth factors following centrifugation due to the reduced relative g-force applied during centrifugation (i.e., less cells were pushed towards the bottom of the centrifugation tubes). When investigating full-sized PRF membranes or plasma layers, this proved to be true and has been confirmed in numerous studies since [37–40]. Furthermore, our group further demonstrated that centrifugation that utilized lower centrifugation speeds

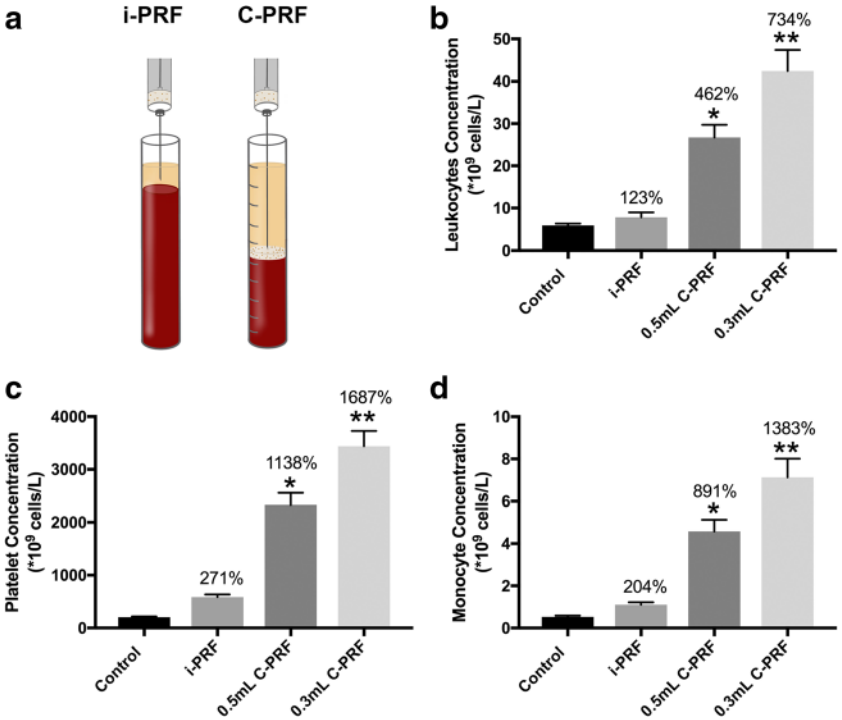

Fig. 5 (**a**) Proposed method to harvest concentrated PRF (C-PRF). Following L-PRF protocols, since all cells accumulated within 300–500 μL above the red cell layer, it was proposed to collect 0.3–0.5 mL of the liquid C-PRF directly above the red cell junction to obtain a liquid plasma layer with highly concentrated platelets and leukocytes. (**b**–**d**) Concentration of (**b**) leukocytes, (**c**) platelets, and (**d**) monocytes following centrifugation using i-PRF protocols versus the collection of 0.3–0.5 mL of concentrated PRF (C-PRF). While i-PRF could typically achieve a 1.2- to 2.5-fold increase in the various cell types following centrifugation, up to a 15-fold increase in the platelet concentration could be achieved with C-PRF. (* represents a significant difference compared to i-PRF; ** represents significantly higher than all groups, $p < 0.05$)

(now termed advanced PRF) released higher levels of total growth factors, such as PDGF, TGF-β1, VEGF, EGF, and IGF, compared to control L-PRF [41, 42].

The findings from the present study represent a shift in our understanding of liquid PRF and the ability to concentrate platelets and leukocytes for liquid injectable use. Previously, work comparing i-PRF and PRP conducted by our group found similar levels of growth factor release between the two protocols, with little understanding of the previously observed results. The findings from the present study represent a breakthrough in understanding the differences reported between PRP, i-PRF, and C-PRF with regard to platelet yields. Many clinicians believe that platelet yields upwards of 1500×10^9 cells/L are ideal for regenerative purposes, yet i-PRF protocols were typically found to isolate cells within the concentration range of $500–600 \times 10^9$ cells/L, and many clinicians deemed this concentration too low. One of the main advantages of i-PRF is that due to its lack of anticoagulants, clotting occurs shortly following injection or during mixing with biomaterials, thus favoring a longer and more gradual release of growth factors over time compared to PRP [41]. Nevertheless, a comparative study between i-PRF and PRP demonstrated very similar growth factor release over time, with some growth factors being released more in i-PRF, whereas others were released in higher levels in PRP [31].

Over the past few months, a sequential pipetting methodology was developed to accurately quantify the effect of centrifugation protocols on the separation of cell layers. This method was developed to accurately identify the location of cells following various centrifugation protocols. While A-PRF protocols, which involve centrifugation at 200 RCF-max (as opposed to 700 in L-PRF protocols), have been shown to more evenly distribute platelets in the upper 4-mL layers of plasma [35], it was interesting to note that a massive accumulation of platelets and leukocytes was observed directly above the red blood cell layer following L-PRF protocols. We therefore aimed to investigate (1) in what volume the majority of these cells were located and (2) what was the maximum concentration of leukocytes and platelets in PRF that could be obtained by harvesting this specific concentrated region of cells (C-PRF).

One of the surprising findings was how concentrated the various cells were in the buffy coat directly above the red blood cell layer following L-PRF protocols. In all samples, cells were routinely situated within 0.3–0.5 mL above the red blood cell corpuscle, with the great majority located 0.1–0.2 mL within the buffy coat above the red blood cell layer. Notably, a large number of leukocytes and monocytes were located in the first 100-μL red blood cell layer, and it will be interesting to determine whether this 100 μL of red blood cells should be collected and included within liquid-PRF formulations due to its large incorporation of various white blood cells. In contrast, although L-PRF has also been referred to as "leukocyte" and platelet-rich fibrin (i.e., L-PRF), it is interesting to note that the leukocyte concentration in L-PRF is actually lower than that what is found in standard whole blood [35]. This means that following the centrifugation protocol to produce L-PRF clots, an actual decrease in leukocyte numbers is observed [35]. The protocol with the greatest ability to concentrate leukocytes (i-PRF) only increases their concentration by approximately 50%. Within the present study, we demonstrated for the first time an ability to concentrate leukocytes between 500 and 750% compared to the concentrations in whole blood by utilizing the C-PRF harvesting method outlined in this study. We also demonstrated the ability to concentrate platelets over 15-fold compared to baseline, whereas i-PRF protocols only concentrate platelets by 2–3-fold (Table 1; Fig. 5). To the best of these authors' knowledge, this is the highest concentration of both platelets and leukocytes observed to date in any PRF preparation.

One interesting phenomenon left to investigate is the possibility of further optimizing the C-PRF protocol. In general, L-PRF protocols are known to concentrate the majority of leukocytes and other white blood cells (greater than 50%) in the red blood cell layer (not included in the PRF layer). Therefore, it would be interesting to further evaluate whether a better yield of platelets and leukocytes could be obtained by further modifying the centrifugation protocols. Another interesting recent development has been the improvements in the ability to separate cell layers (based on density) using a horizontal centrifuge which resulted in up to a 4-fold increase in cell concentrations [35]. Future studies comparing horizontal centrifugation versus fixed-angle centrifugation for the harvesting of C-PRF is therefore needed. In summary, the findings from the present study report the development of a novel method to harvest the enriched portion (more platelets/leukocytes) directly from the liquid layer directly above the red blood cell layer within the buffy coat for the collection of a liquid injectable PRF highly rich in platelets and leukocytes.

Conclusion

The present study describes a novel technique for investigating the cell accumulation directly above the red blood cell layer within the buffy coat. A massive accumulation of leukocytes, platelets, and monocytes was located specifically within this 0.3- to 0.5-mL layer when utilizing high g-force protocols (L-PRF). While conventional i-PRF protocols are able to concentrate leukocytes up to 1.5-fold and platelets 2–3-fold, we demonstrated that there was a > 500% increase in leukocytes and a > 1500% increase in platelets when utilizing this novel concentrated PRF (C-PRF) harvesting technique. Further comparative preclinical and clinical studies are now needed to examine the added regenerative potential of C-PRF compared to conventional i-PRF protocols.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval No ethical approval was required for this study, as the human samples were not identified.

Informed consent For this study, informed written consent was provided to conduct the outlined experiments prior to the blood draw.

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