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ORIGINAL ARTICLE

A novel method for evaluating and quantifying cell types in platelet rich fibrin and an introduction to horizontal centrifugation

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Abstract

Platelet rich fibrin (PRF) has been utilized clinically as a platelet concentrate capable of stimulating tissue regeneration. Interestingly, several protocols have been proposed with little data obtained regarding the final cell counts following centrifugation. The aim of the present study was to compare different commercially available centrifuges and their respective protocols utilizing a novel method to quantify cells. One millimeter blood layers following centrifugation were sequentially pipetted from the upper layer downward until all 10 mL were harvested in sequential samples. Thereafter, each sample was sent for CBC analysis to accurately quantify precisely cell numbers within each separate blood layer following centrifugation. The results from this study revealed that L-PRF protocols (2700 rpm × 12 min) produced a clot with the majority of platelets and leukocytes concentrated within the buffy coat with relatively no cells found within the first 4 mL of L-PRF. Slower centrifugation protocols produced using the A-PRF protocols (1300 rpm × 8 min) produced a more evenly distributed number of platelets throughout PRF. Injectable-PRF (i-PRF) protocols produced the highest concentration of leukocytes/platelets, however, the total number of leukocytes and platelets were significantly lower owing to the decreased total volume collected. Horizontal centrifugation produced a significant increase in both the number and concentration of platelets and leukocytes (up to 3.5× higher for either solid/liquid PRF). When compared to either fixed or angled centrifuge (InstraSpin, Process for PRF). In conclusion, the present study revealed a novel/accurate method to quantify cells following PRF protocols. Furthermore, PRF produced via horizontal centrifugation accumulated a higher number and concentration of platelets/leukocytes when compared to either fixed-angle centrifugation.

KEYWORDS
blood platelets, centrifugation, fibrin, platelet rich fibrin

1 | INTRODUCTION

Platelet concentrates have been widely utilized in many fields of medicine owing to their ability to rapidly stimulate vascularization of tissues (Miron et al., 2017). Platelet-rich plasma (PRP) was first introduced over 20 years ago following studies demonstrating the role of platelets during tissue regeneration (Cai, Zhang, & Lin, 2015; Marx, 2004; Marx et al., 1998; Meheux, McCulloch, Lintner, Varner, & Harris, 2016;

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Singh & Goldberg, 2016). PRP contains an array of autologous growth factors including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-beta), vascular endothelial growth factor (VEGF) among others, responsible for the migration and/or proliferation of various cell types (Cai et al., 2015; Marx, 2004; Marx et al., 1998; Meheux et al., 2016; Singh & Goldberg, 2016). While PRP has been widely successful in many fields of medicine and continues to be widely utilized, reports regarding its inclusion of anti-coagulants raised concerns since clotting are an important step during wound healing (Anfossi et al., 1989; Fijnheer et al., 1990; Marx, 2004). Nevertheless, the ease of harvesting peripheral blood and concentrating blood-derived growth factors using centrifugation have long been considered a low-cost and easy-to-obtain source of natural growth factors (Chow, McIntire, & Peterson, 1983; Delaini, Poggi, & Donati, 1982).

Following the development of PRP, the second-generation platelet concentrate was pioneered with the aim of removing anti-coagulants. This was termed leukocyte and platelet rich fibrin (L-PRF) with a most commonly utilized protocol of ~700g for 12 min. Since anti-coagulants were removed, blood is subject to clotting over time and it therefore becomes necessary for the clinician to begin centrifugation shortly following blood collection to separate blood layers prior to clotting. Following centrifugation, a fibrin clot is obtained in the upper "platelet-rich" layer (Ehrenfest, Rasmusson, & TJTib, 2009). Typically, cells contained within this layer include platelets and leukocytes entrapped within this fibrin matrix. The main role of the fibrin matrix has since been described to favor the slow and gradual release of growth factors over time when compared to PRP (Kobayashi et al., 2016).

More recently, it has been found that lower centrifugation speeds and times release a higher amount of growth factors over time (Kobayashi et al., 2016). It has therefore been reported in the literature that high centrifugation speeds tend to push cells toward the bottom of PRF tubes, where it was revealed that slower centrifugation speed (from a relative centrifugal force [RCF]-max of ~700g to ~200g) resulted in a PRF matrix with more concentrated cells and growth factors throughout the PRF matrix (Choukroun & Ghanaati, 2018; El Bagdadi et al., 2017; Fujioka-Kobayashi et al., 2017; Ghanaati et al., 2014; Ghanaati et al., 2018; Kobayashi et al., 2016; Kubesch et al., 2018). This work led to the development of advanced platelet rich fibrin (A-PRF) with a protocol of ~200g for 8 min when compared to original protocols of ~700g for 12 min (L-PRF protocols) (Choukroun & Ghanaati, 2018; El Bagdadi et al., 2017; Fujioka-Kobayashi et al., 2017; Ghanaati et al., 2014; Ghanaati et al., 2018; Kobayashi et al., 2016; Kubesch et al., 2018). Furthermore, by further reducing spin protocols, a liquid-PRF (injectable-PRF or i-PRF) was described as having an increased concentration of platelets and leukocytes by performing a short centrifugation protocol (~60g for 3 min) (Miron et al., 2017).

Over the past few years, several additional commercially available centrifuges have been brought to market. These vary in many factors including protocols, RCF-values, tube-rotor angulation, rotor radius size and tube composition. Each of these plays a role in the final obtained PRF membrane yet little data are scientifically available displaying cell numbers and content in the various layers following centrifugation. Furthermore, while horizontal centrifugation has been utilized frequently for the production of PRP, to date, no commercially available horizontal centrifuge has been brought to market for the production of PRF, though in theory it offers a better ability to separate cell types based on density owing to its greater difference in RCF values between the RCF-min and RCF-max (Lourenço et al., 2018).

While much commercial debate exists on the topic, there also exists no accurate method to quantify/determine the precise location of cells following centrifugation with few histological studies performed investigating cell numbers within the fibrin clots. In the present study, we proposed a novel method to quantify cell numbers and concentration within the PRF scaffolds following centrifugation by utilizing a sequential pipetting methodology. First, liquid-PRF and solid-PRF protocols were utilized and produced on three centrifuges according to their respective manufacturer's protocols. Following centrifugation, 1 mL layers were sequentially pipetted from the upper layer of blood tubes toward the bottom of the tube until all 10 mL were harvested in sequential samples. Ten samples from each centrifugation tube was then sent for complete blood count (CBC) analysis to accurately quantify precisely cell numbers within each of the 10 mL blood layers (1 mL at a time) and then compared according to cell numbers and concentrations. This study represents a novel experimental methodology to more accurately depict cell numbers in PRF following centrifugation using various protocols.

2 | MATERIALS AND METHODS

2.1 | Preparation of PRF

Blood samples were collected with the informed consent from six volunteer donors. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. No ethical approval was required for this study because human samples were not identified, as previously described (Miron, Fujioka-Kobayashi, et al., 2017). The factors that affect fibrin clot formation and structure include genetic factors, acquired factors (such as abnormal concentration of thrombin and factor XIII in plasma, blood flow, platelet activation, oxidative stress, hyperglycemia, hyper-homocysteinemia, medications, and cigarette smoking), and other parameters (such as microgravity, pH, temperature, reducing agents, and concentration of chloride and calcium ions) (Nunes, Roedersheimer, Simske, & Luttges, 1995). All patients with any of the above conditions were excluded. All patients were included if systemically healthy, non-smoking, and not taking any medications.

The following three centrifugation devices were utilized in this study including the IntraSpin Device (IntraLock, Boca Raton, Florida), The Duo Quattro (Process for PRF, Nice, France) and an Eppendorf horizontal centrifuge (Eppendorf 5702 centrifuge, Germany). Two separate protocols were tested on each machine including the manufacturer's recommendation to produce both liquid- and solid-PRF. On the Intraspin device, leukocyte and platelet rich fibrin (L-PRF) protocol (~700 RCF-max [~400 RCF-clot] for 12 min) were utilized for the

solid-PRF clot whereas a ~700 RCF-max for 3 min was utilized to produce liquid-PRF. On the process for PRF device, the advanced platelet rich fibrin (A-PRF) protocol (~200g RCF-max for 8 min) was utilized to produce solid-PRF and a liquid-PRF was produced using the i-PRF protocol of ~60g RCF-max for 3 min. A horizontal centrifuge was utilized owing to its advantage in separating layers based on density. Two protocols were utilized in this study including a solid-PRF protocol of 700g for 8 min and a liquid-PRF protocol of 200g for 8 min. In total six protocols were utilized and described according to previous literature (Miron, Choukroun, Ghanaati, & SCi, 2018; Miron, Pinto, Quirynen, & **SJJop**, 2019).

Each of the six volunteers donated two blood collection tubes (10 mL plastic tubes) for each of the six tested groups for a total of 12 tubes per participant and an additional one blood sample that acted as a control. Each pair of tubes was utilized to balance the centrifuge during the spin cycle. The first tube was sequentially investigated from the top 1 mL layer down to the bottom 1 mL layer as depicted in Figure 1. Noteworthy, during the harvesting of 1 mL layers, for each centrifugation protocol, one sample was collected between the plasma/buffy coat and red blood cell layer (Figure 2). This layer was marked within each figure to represent the location of the buffy coat and displayed graphically in Figures 4–9 with arrows to represent the separation between the yellow plasma and red blood cell layers.

The second tube from each participant was utilized to determine the final concentration from both the yellow plasma layer and the red blood cell layer. Furthermore, total volumes (in mL) were recorded from each layer to calculate total cell numbers and concentrations in each layer. Blood draw was carried out with anti-coagulants (2 mg/mL EDTA-K2) to allow for blood samples to thereafter be sent for complete blood counts (CBC) where the total number of leukocytes, red blood cells, platelets, neutrophils, lymphocytes and monocytes were calculated from each sample using a Sysmex XN-550 (Sysmex Corporation, Kobe, Japan) based on fluorescence flow cytometry. Thereafter, each sample was displayed graphically using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA).

3 | RESULTS

3.1 | Cell numbers following centrifugation using various protocols/devices

Following centrifugation utilizing the various protocols, 1 mL sequential layers were sent for CBC analysis according to Figure 1. In order to better understand trends following centrifugation using these various protocols/devices, the data were purposely not averaged between patients to better understand the exact separation of blood layers. Instead, each patient was investigated individually and a representative patient sample is displayed in Figures 3–9 (same patient with the same baseline blood counts) following centrifugation using the centrifugation protocols proposed to demonstrate the advantage of this novel technique to determine the precise location of blood cell types following centrifugation.

In Figure 3, the total volume of the plasma layer was noted following each protocol. In general, the solid PRF protocols generated larger separations owing to higher RCF and time when compared to the

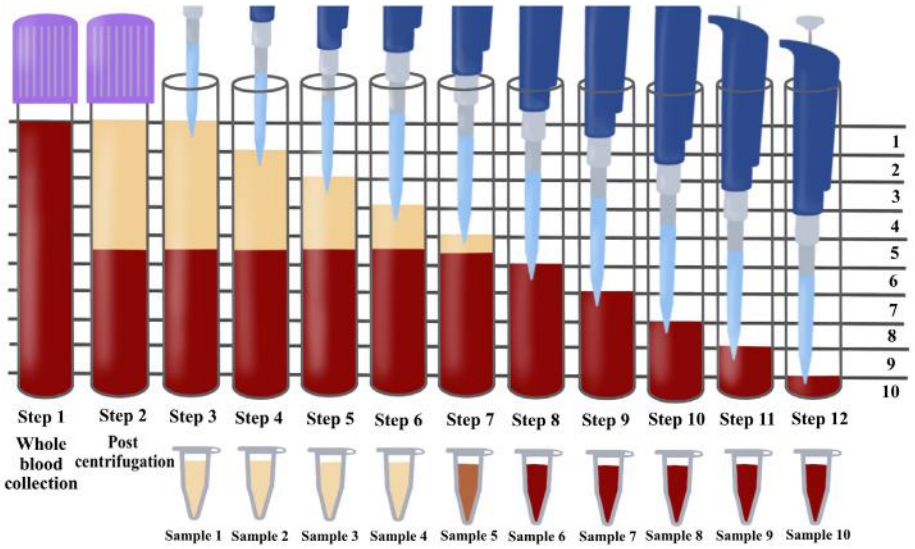

FIGURE 1 Illustration demonstrating the proposed novel method to quantify cell types following centrifugation of PRF. Currently, one of the limitations is that whole blood is compared to the total plasma concentration following centrifugation. This however, does not give a proper representation regarding the location of cells following centrifugation. By utilizing the proposed technique in this study by sequentially pipetting 1 mL of volume from the top layer downward, it is then possible to send each of the 10 samples for CBC analysis and accurately determine the precise location of each cell type following centrifugation at various protocols. Notice that 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 is typically located. PRF, platelet rich fibrin

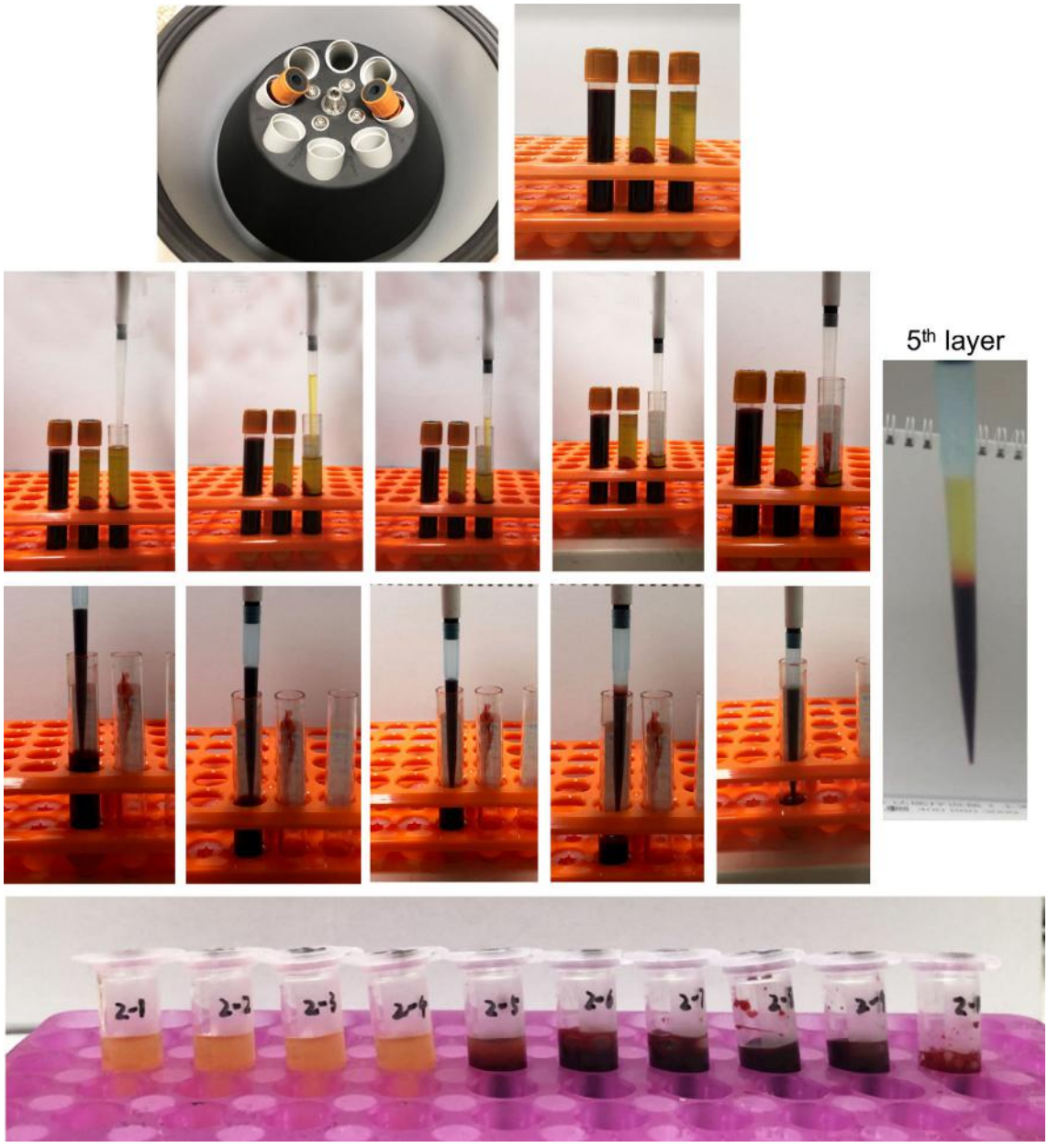

FIGURE 2 Visual demonstration of the protocol. Following centrifugation with two 10 mL centrifugation tubes, blood layers are then separated. Thereafter, 1 mL samples are pipetted precisely from the upper layer downward. Notice that when layer 5 was drawn, it was possible to visualize the layer separation between the yellow plasma and red blood corpuscle layers. This separation layer was noted for all samples

shorter spin cycles/RCF values produced for liquid PRF. In Figure 4, the original L-PRF protocol is displayed utilizing a 2,700 rpm (~700g) for 12 min protocol using a 33° fixed-angle centrifuge. Notice the average number of cells displayed for each cell type in the control bar for whole blood (Figure 4). This methodology revealed precisely that the number of leukocytes (control 6×10^9 cells/L) was significantly concentrated in layer 5 (~17×10^9 cells/L). The arrows represent the buffy coat layer between the yellow plasma and red blood cell layers (Figure 2). Interestingly, a three–fourfold increase in leukocyte number was observed specifically at this interface within the buffy coat. Notice, however, that no leukocytes were found in any of the first 4 mL layers, displaying a very uneven PRF clot with respect to cell numbers. Almost all cells within the PRF clot were exclusively found within this fifth layer. Notice also that more leukocytes were found in the red blood cell layer below the PRF clot. A similar trend was also observed for lymphocytes, neutrophils and monocytes. Naturally all red blood cells were found in layers 5 through 10 in the visually red layers. Platelets were accumulated precisely in layer 5 (six–eightfold), within the "buffy coat." Since the majority of cells were found in layer 5, we were then interested to determine if these cells were specifically found within the yellow plasma layer (within the PRF clot) or within the red blood cell layer. For this, the second blood tube was

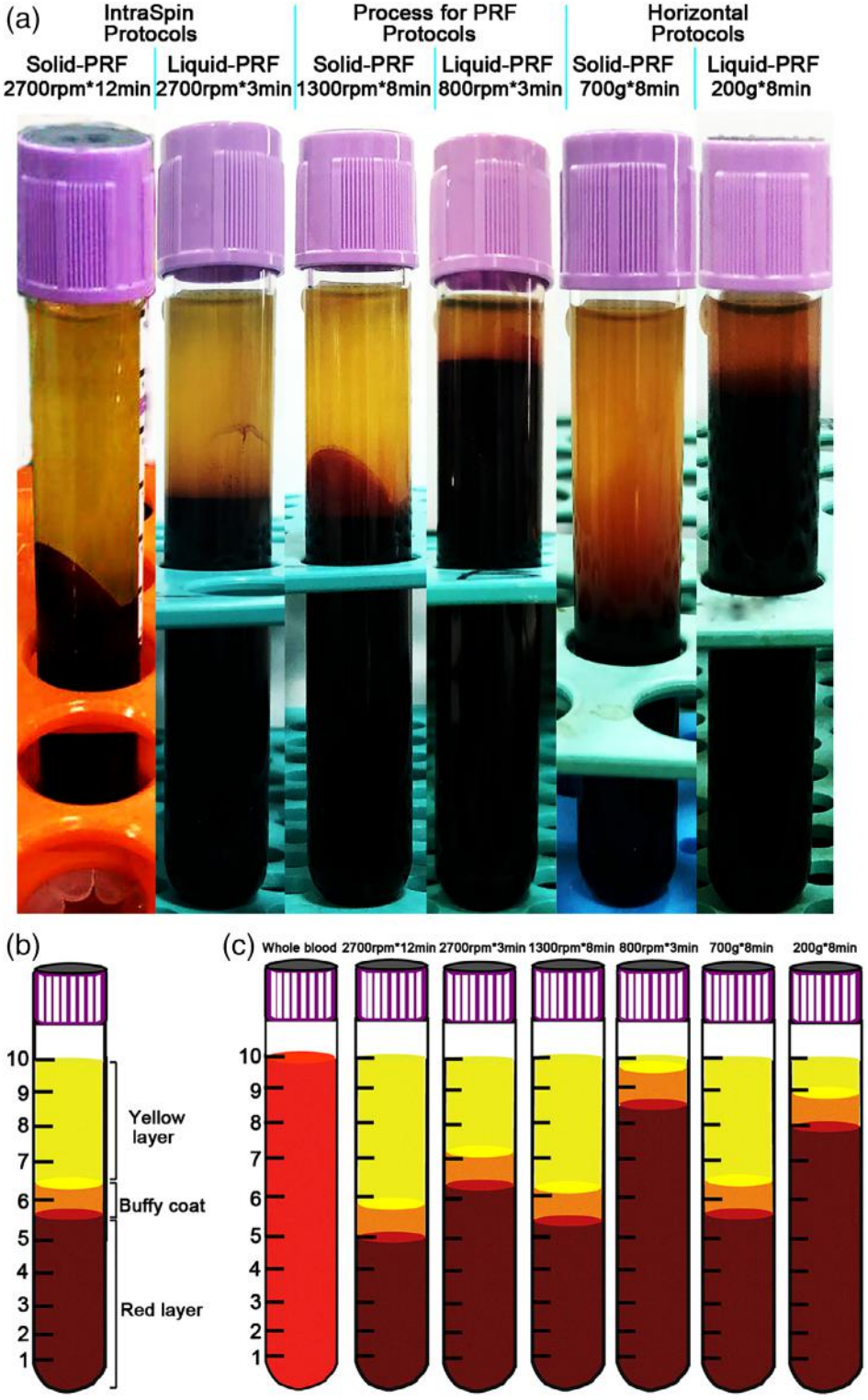

FIGURE 3 Visual representation of the layer separation following centrifugation utilizing the appropriate manufacturer-recommended protocol. Notice that on the fixed-angle centrifuges include the IntraSpin (2700 rpm for 12 min) and process for PRF (1300 rpm for 8 min), an angle red blood layer is always observed owing to the angle centrifugation process. Notice that by utilizing horizontal centrifugation, a completely perpendicular separation is observed. PRF, platelet rich fibrin

utilized and 500ul of blood volume was collected just above the red blood layer within the buffy coat, and 500μl was taken from the red blood cell layer. It was revealed that the majority of platelets were found within the yellow plasma layer (>80%) whereas the majority of leukocytes and other white blood cells were found within the red blood layer (Figure S1). This revealed that most leukocytes were in fact not found within the PRF layers utilizing the L-PRF protocol.

The final concentrations of each centrifugation protocol are depicted in Table 1. Interestingly, the final concentration of leukocytes found using the L-PRF protocol was 4.13×10^9 cells/L whereas original control values from this patient was 6.125; representing 67%

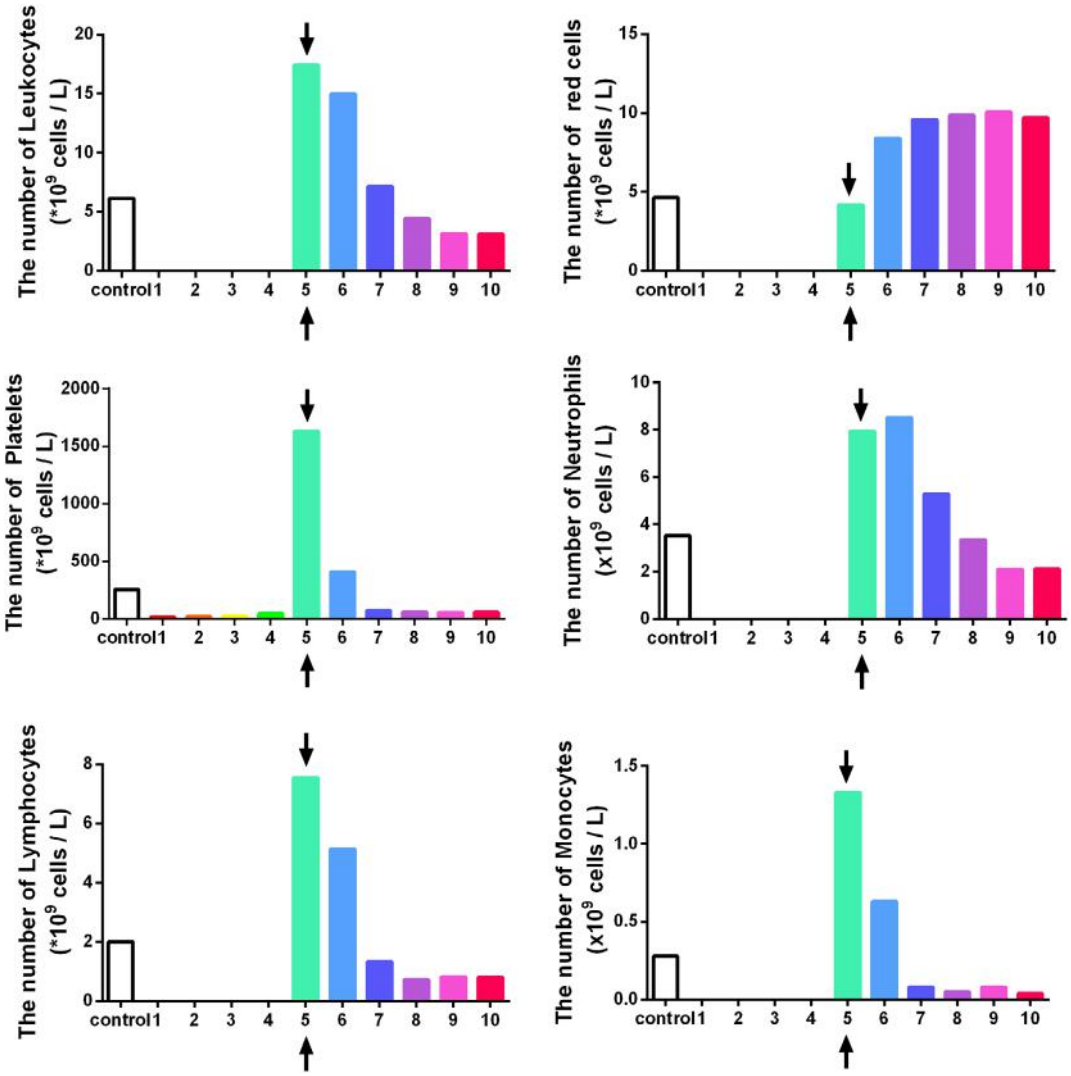

FIGURE 4 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the solid L-PRF protocol (2700 rpm for 12 min; ~700g). Notice that the majority of platelets accumulated directly within the fifth layer in the buffy coat. Furthermore, the highest concentration of leukocytes was also noted in this layer. The first four layers of this plasma layer, was typically devoid of all cells. PRF, platelet rich fibrin

of the original values (a reduction in leukocytes when compared to control blood). Platelet numbers were increased 1.61 fold. The total leukocyte and platelet content represent 33 and 80% of the total blood cells found, respectively, within this 10 mL blood sample. Therefore, it may be concluded that the majority of leukocytes were in fact not found within the plasma layer utilizing the L-PRF protocol and that the majority of platelets found utilizing this protocol were found specifically in layer 5 within the buffy coat (Figure 4).

Figure 5 depicts centrifugation following A-PRF protocols (1,300 rpm for 8 min on a Process for PRF centrifuge). Interestingly, the number of platelets was concentrated throughout the first 4–5 layers, unlike the L-PRF protocol. Here a twofold increase in platelets was observed compared to a 1.6 fold increase utilizing the L-PRF protocol. More importantly, however, the platelets were found evenly distributed throughout the A-PRF plasma layers. When investigating leukocyte number however, a significantly lower concentration (33% original values) as well as total numbers 9.315 versus 20.65×10^9 cells/L were found in the A-PRF group when compared to L-PRF. Therefore, it may be concluded that either (a) the g-force or (b) the total time was not sufficient for adequately accumulation/separation of leukocytes utilizing the A-PRF protocol (Figure 5). Only 15% of the total leukocytes found in blood were actually accumulated within the 4.5 mL A-PRF volume.

Figure 6 depicts a 700g force performed on a horizontal centrifuge for 8 min. Interestingly, it was observed that more leukocytes, platelets, lymphocytes, and monocytes were more evenly distributed throughout the PRF layers when compared to fixed-angle centrifugation. Unlike either the L-PRF or A-PRF protocols, a general increase in leukocyte numbers was observed (127% original values) and a 2.4 fold increase in platelet concentration was observed. This represents over

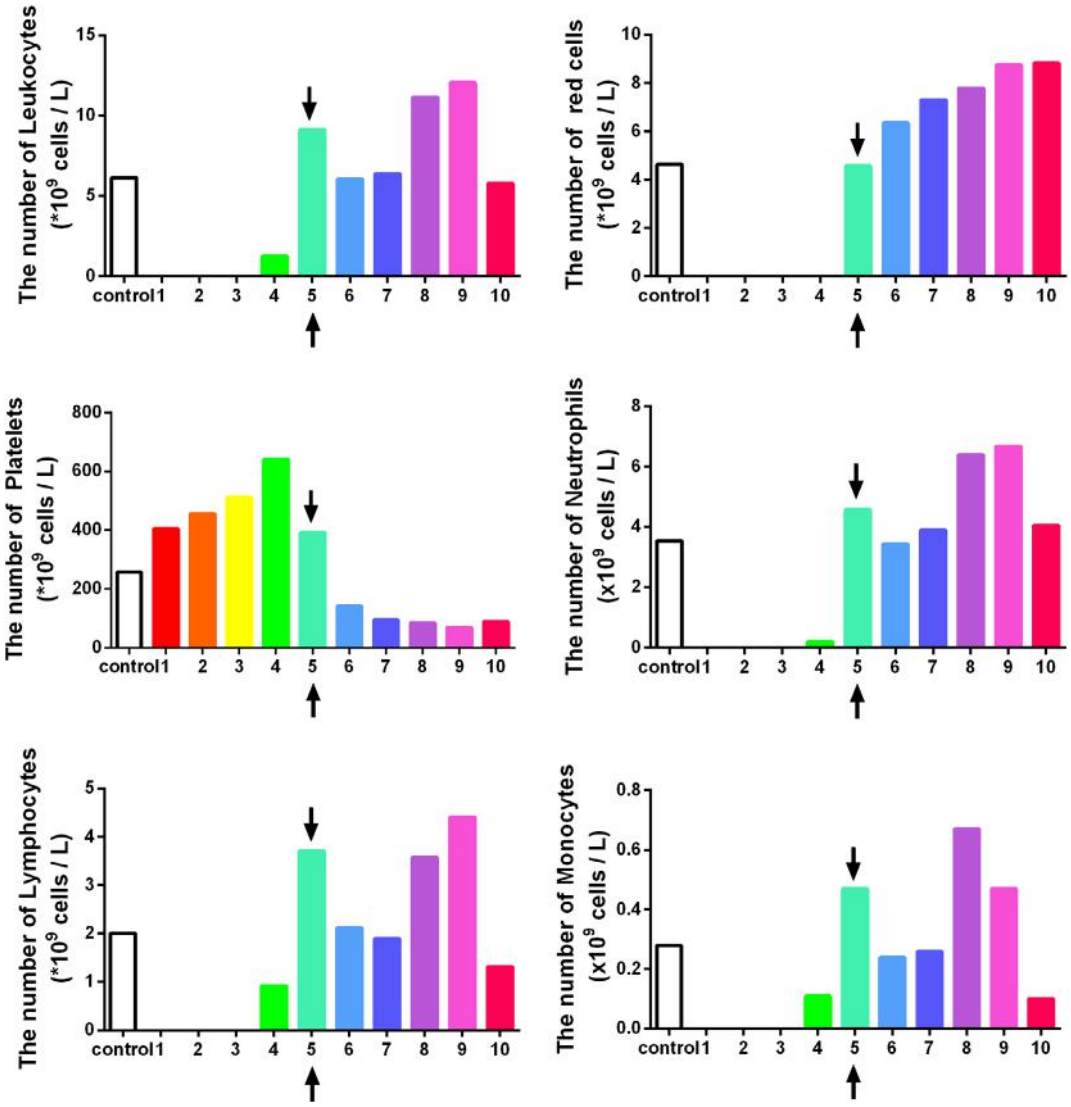

FIGURE 5 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the solid A-PRF protocol (1300 rpm for 8 min; ~200g). Notice that specifically the platelets were more evenly distributed throughout the upper 5 mL plasma layer. Noteworthy however, the majority of white blood cells (leukocytes, neutrophils, lymphocytes, and monocytes) were not found in the upper plasma layer. PRF, platelet rich fibrin

a fourfold increase in leukocytes when compared to A-PRF protocols and a twofold increase when compared to L-PRF (Table 1). Furthermore, this method concentrated 99.7% of all platelets and 53% of all leukocytes within the plasma layer.

Thereafter, liquid PRF protocols were investigated and compared (Figures 7–9). The InstraSpin protocol (2,700 rpm for 3-min protocol [~700g]) was first represented in Figure 7. Interestingly, this protocol accumulated platelets evenly throughout the PRF layer better than when utilizing the 12-min protocol. Nevertheless, leukocytes were significantly lower once again when compared to whole blood representing only 54% of the original control blood concentrations. This demonstrates that following centrifugation, ***lower*** leukocytes are found in L-PRF samples when compared to control blood. Platelet concentrates were increased 2.12 fold representing the highest concentration of platelets when compared to all other fixed-angle centrifuges.

The injectable-PRF protocol produced a 1.23 fold increase in leukocyte concentration and a 2.07 fold increase in platelet concentration when compared to whole blood (Figure 8). The overall accumulation demonstrated an 18% total leukocyte content and a 31% total platelet count when compared to whole blood (Tables 1 and 2). This represented an extremely low platelet yield as all other protocols produced at least 80% total yield (Table 1). Most notably, the change in cell density layer by layer, as depicted in Figure 8 was almost unnoticeable. Therefore, the findings revealed that the i-PRF protocol displayed an inability to concentrate cells effectively.

The final protocol producing liquid PRF on a horizontal centrifuge demonstrated the highest concentration of leukocytes 10.92×10^9

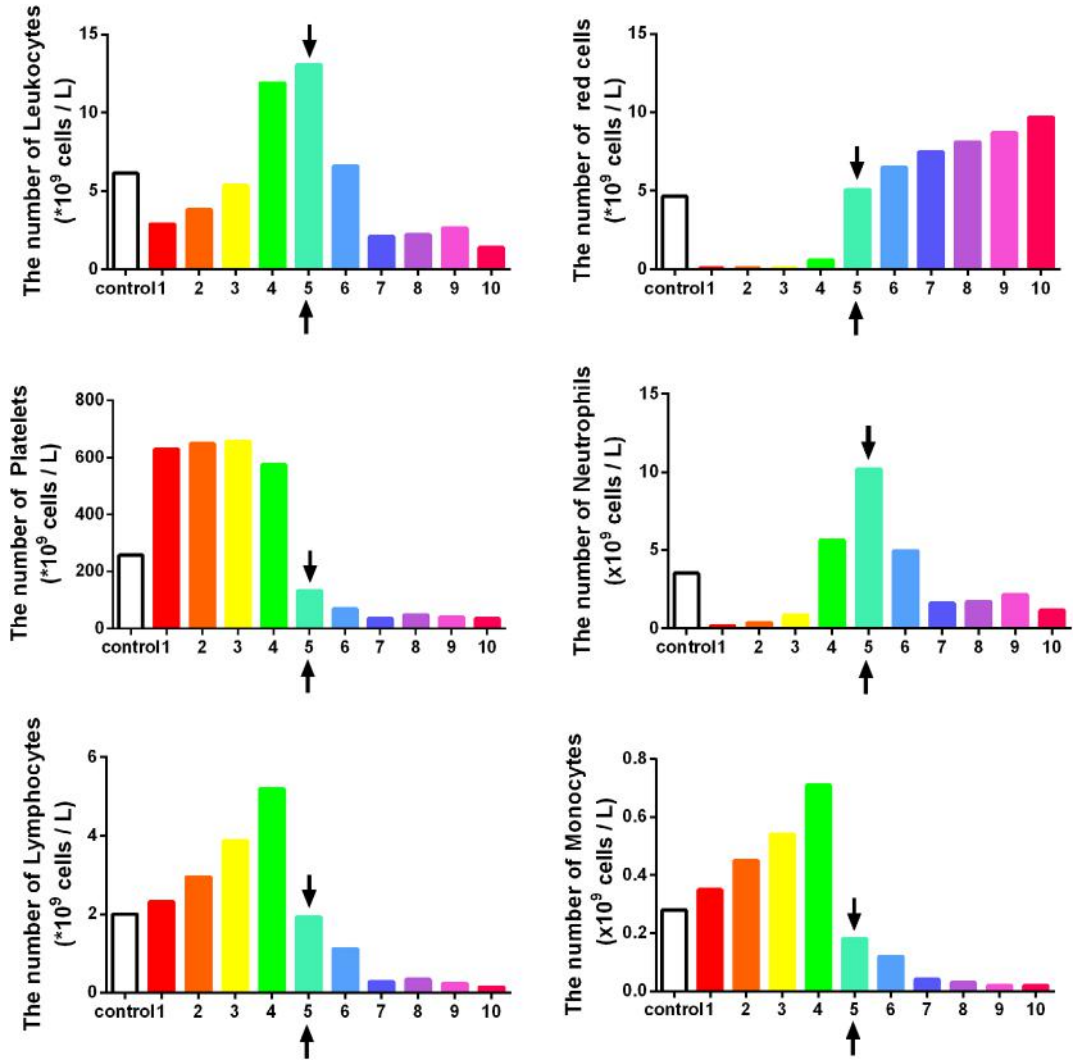

FIGURE 6 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the solid-PRF horizontal centrifugation protocol (700g for 8 min). Notice that most of the platelets as well as white blood cells are now more evenly distributed throughout the upper plasma layer. PRF, platelet rich fibrin

cells/L (178% original values) and the highest concentration of platelets when compared to all other groups (Figure 9). This protocol demonstrated the most effective method to concentrate leukocytes, platelets, and monocytes when compared to all other methods (Table 1).

Figure 10 demonstrates a representative bar graph from each protocol summarizing the final concentrations of platelets and leukocytes as well as their total numbers within their layers (including % of control and % total yield). Notice that the horizontal centrifuge produced the highest number of leukocytes and platelets with approximately a twofold increase in leukocyte numbers when compared to all other protocols produced on a fixed-angle centrifuge.

4 | DISCUSSION

PRF has gained tremendous momentum in recent years as a regenerative agent capable of promoting tissue regeneration. Despite its widespread use, it is interesting to note that to date very little scientific data exists investigating the various protocols that are clinically recommended by various manufacturers. In the year 2014, Ghanaati et al. proposed lower centrifugation speeds as a means to better accumulate growth factors and cells within the upper platelet-rich layers. In our study, we also demonstrated that roughly a 20% increase in platelet concentration was observed following these lower speed centrifugation protocols (Ghanaati et al., 2014). We also confirmed that even lower centrifugation protocols (~60g for 3 min) was responsible for higher concentrations of leukocytes and platelets (Table 1). While these previous methods based on histological observation allowed for a rough estimate of the cells found in the various layers, the new methodology proposed in the present study allows for the precise location and concentration of cells in 1 mL incremental layers following centrifugation. This allowed for a better understanding of the events occurring during centrifugation and an array of future research is now possible as a result.

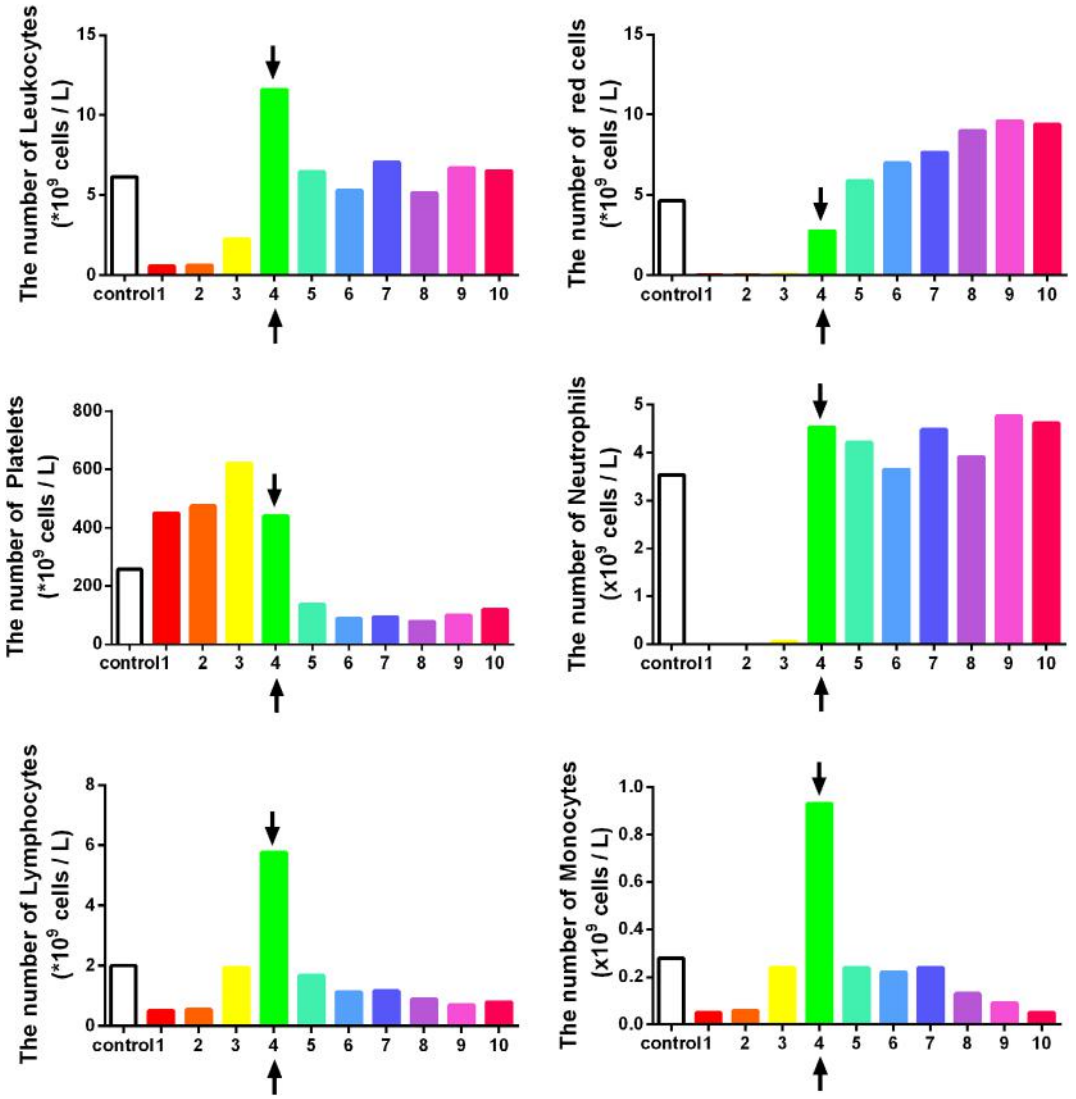

FIGURE 7 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the liquid-PRF IntraSpin protocol (2700 rpm for 3 min; ~700g). Notice that most platelets are more evenly distributed utilizing this protocol when compared to the 12 min solid-PRF IntraSpin protocol. PRF, platelet rich fibrin

One of the first interesting and surprising findings was the observation that almost all platelets were accumulated in layer 5 using the conventional L-PRF protocol. Almost no platelets were observed in the first four layers following centrifugation and the majority of leukocytes were found in the red cell layer, not included in the PRF clot. This was a bit ironic, granted the working tradename "leukocyte" and platelet rich fibrin (or L-PRF). Previous studies have shown that L-PRF protocols result in lower platelet and leukocyte numbers when compared to various other protocols produced at lower RCF values (Choukroun & Ghanaati, 2018; Ghanaati et al., 2014). While platelet concentrations were also lower in the present study, we revealed that the actual differences were not as drastic as previously reported (Choukroun & Ghanaati, 2018). Interestingly, our study demonstrated more specifically that the platelets and leukocytes are in fact found ***precisely*** located at the junction/border between the yellow and red cell interface. Likely these previous studies failed to collect ***all*** liquid within the yellow plasma layer and as a result, extremely low platelet and leukocyte values may have been reported. As clearly shown in the present study, failure to do so, especially at a g-force of 700g or greater, results in extremely low concentration values since the upper 4 mL of plasma is practically devoid of cells (Figure 4). Therefore, we demonstrate in the present study that L-PRF protocols are in fact quite rich in platelets, however, these cells are found within a 1 mL layer directly above the red blood corpuscles within the buffy coat. It also demonstrates the effectiveness of the present methodological protocol applied for evaluating PRF protocols.

In the present study, the A-PRF protocol resulted in a more evenly distributed platelet layer throughout the PRF layer further validating the low-speed centrifugation concept. Lower g-forces centrifuged for less time consistently resulted in a better distribution of platelets throughout the PRF matrix in the upper 4–5 mL whereas an uneven distribution of cells was found using the 700g by 12-min protocol. It is

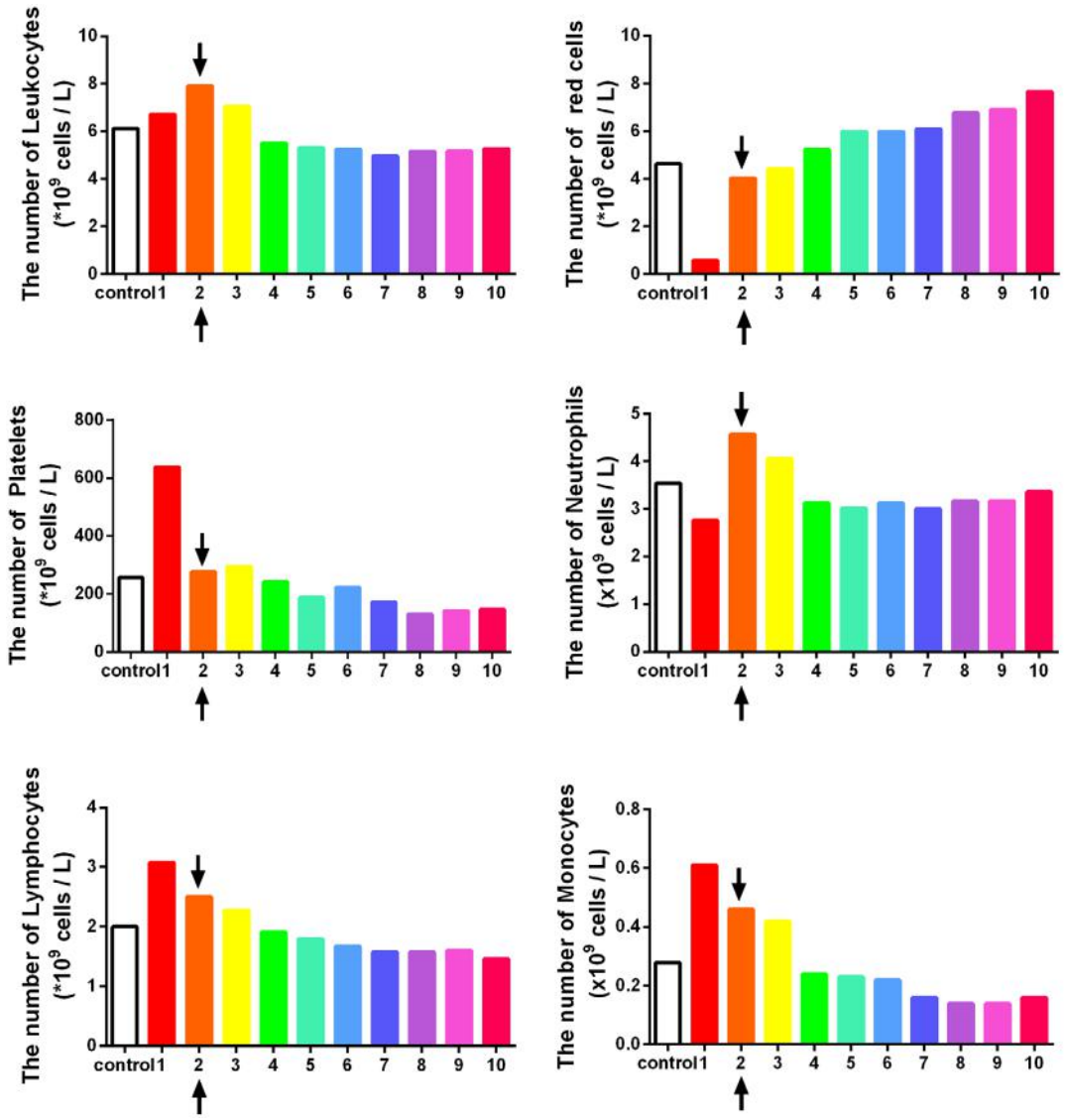

FIGURE 8 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the liquid-PRF process for PRF (i-PRF) protocol (800 rpm for 3 min; ~60g). Notice that very little change in platelet or leukocyte accumulation is observed utilizing this centrifugation cycle. A slight increase in platelets and leukocytes is however observed when compared to control. PRF, platelet rich fibrin

therefore clinically recommended avoiding utilizing original L-PRF protocols for membrane fabrication as all the cells are entirely found in a thin layer at the base of the PRF clot. It was however, interesting to note that low leukocyte yields were observed utilizing these protocols.

It was also interestingly observed that the Process for PRF manufacturer's recommended protocol for liquid PRF (injectable-PRF at ~60g for 3 min) was neither adequately effective at separating cell types or producing high yields of platelets/leukocytes. Figure 8 demonstrated minimal change in cell numbers following this short centrifugation cycle at low RCF values. This protocol even demonstrated a slightly inferior platelet concentration when compared to the liquid L-PRF protocol (~700g for 3 min) with a ~2.5 fold decrease in total platelet yield owing to the decreased volume. Based on the data obtained within this study, a paradigm shift in our understanding of platelet concentrates with respect to the low speed centrifugation concept is noted. It therefore becomes possible to simply centrifuge at too low RCF-values/times to produce ineffective separation of blood layers as demonstrated in i-PRF protocols.

With respect to previously published data on the topic, it remains interesting to point the pitfalls to previously utilized methods to quantify PRF as the majority of cells utilizing higher speed centrifugation accumulate directly over the red blood layer (within the buffy coat). Failure to collect this minute layer (~300 μL) (especially utilizing L-PRF protocols) results in a major loss in cell numbers.

Within the present study, horizontal centrifugation was proposed as a means to better separate cell layers based on density. Previously, Lourenço et al. have demonstrated the ability to produce PRF via horizontal centrifugation (Lourenço et al., 2018). Two advantages are noted utilizing horizontal centrifugation. First, a completely horizontal

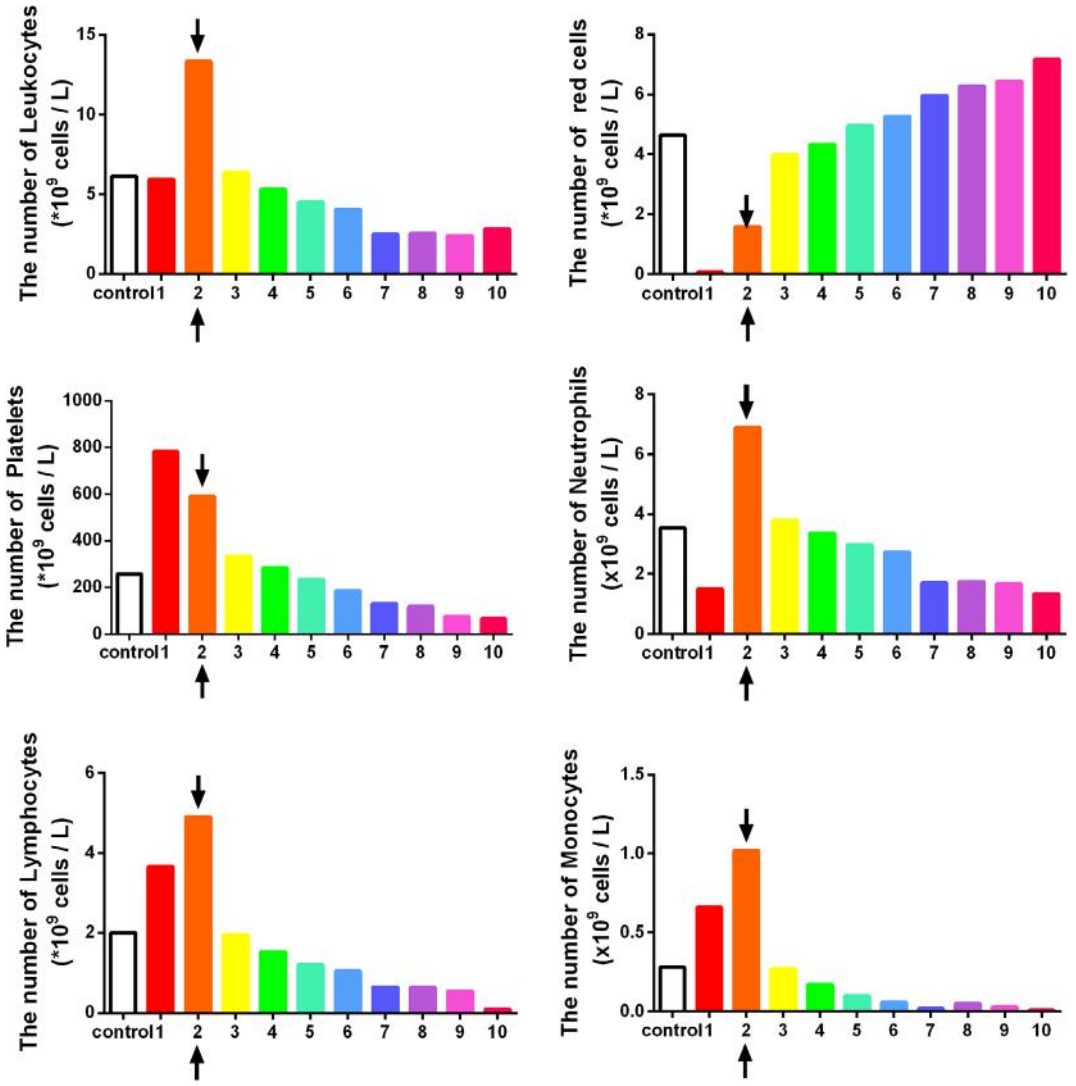

FIGURE 9 The concentration of cell types in each layer from 1 mL down to the 10th mL sample utilizing the liquid-PRF horizontal centrifugation protocol (200g for 8 min). Notice that most this protocol produced the highest concentration of platelets and leukocytes when compared to all other centrifugation protocols. PRF, platelet rich fibrin

tube produced from a swing-out bucket allows for the greatest differential between the minimum and maximum radius found within a centrifugation tube (Figure 11a). This allows for a greater ability to separate cell layers based on disparities between the RCF-min and RCF-max produced within a tube. Secondly, a fixed-angle centrifuge results in more trauma to cells. Since centrifugation typically pushes cells outward and downward, cell layer separation is always observed in an angulated fashion using fixed-angle centrifuges (Figure 11b). These were also noted in the present study in the separation of layers in Figure 3 for the L-PRF and A-PRF protocols where the accumulation of red blood cells is found angled on the tube walls owing to the use of a fixed-angle centrifugation. Furthermore, during the centrifugation process, cells are pushed toward the outer wall and are then typically migrate either up or down the centrifugation tube based on density. It is hypothesized that larger cells (such as red blood cells) entrap smaller cells such as platelets during the centrifugation process and drag them downward along the back centrifugation wall into the red blood cell layer as a result of cell accumulation against the back wall (Figure 11c). In contrast, PRF produced via horizontal separation allows the direct flow through of cells (Figure 11c). Therefore, horizontal centrifugation allows cells to migrate freely throughout the blood layers. This allows for better separation of cell types (along with the greater differentiation in RCF values between RCF-min and RCF-max) resulting in a higher final concentrations of desired cells (platelets and leukocytes) within the appropriate final blood layers. Furthermore, cells are less likely to suffer potential damage along the back wall of centrifugation tubes produced using high g-forces (~200–700g forces) following fixed-angle centrifugation. We therefore introduce this concept as "gentle centrifugation" whereby cells are more freely able to separate between layers without the necessary friction produced on the back wall of fixed-angle centrifuges such as those produced on the fixed-angle IntraSpin and Process for PRF devices.

Interestingly, in the present study, a two to fourfold increase in leukocytes was also found utilizing horizontal centrifugation when compared

TABLE 1 Total volume of plasma (mL), leukocyte concentration, percentage of total leukocytes when compared to whole blood, platelet concentration, percentage of control platelets, monocyte concentration and percentage of monocyte concentration

Group	Protocol	Total volume of plasma (mL)	[leukocytes] 10ˆ9/L	%control - [leukocytes] (6.125 × 10ˆ9/L)	[platelets] 10ˆ9/L	%control - [platelets] (257 × 10ˆ9/L)	[monocytes] 10ˆ9/L	%control - [monocytes] (0.28 × 10ˆ9/L)
Solid-PRF (IntraSpin)	2700 rpm × 12 min (~700g)	5	4.13	67.4	415	161.5	0.34	121.4
Solid-PRF (process)	1300 rpm × 8 min (~200g)	4.5	2.07	33.8	505	196.5	0.14	50.0
Solid-PRF (horizontal)	700g × 8	4.2	7.78	127.0	610	237.4	0.58	207.1
Liquid-PRF (IntraSpin)	2700 rpm × 3 min (~700g)	3.7	3.33	54.4	546	212.5	0.29	103.6
Liquid-PRF (process)	800 rpm × 3 min (~60g)	1.5	7.53	122.9	531	206.6	0.68	242.9
Liquid-PRF (horizontal)	200g × 8	2	10.92	178.3	641	249.4	0.96	342.9

Abbreviation: PRF, platelet rich fibrin.

TABLE 2 Total volume of plasma (mL), leukocyte number, percentage of leukocytes number when compared to whole blood, platelet number, percentage of platelet numbers, monocyte number, and percentage of total monocyte numbers

Group	Protocol	Total volume of plasma (mL)	Total number of leukocytes (10ˆ6)	%Total leukocytes (61.25 × 10ˆ6)	Total number of platelets (10ˆ6)	%Total platelets (2570 × 10ˆ6)	Total number of monocytes (10ˆ6)	%Total monocytes (2.8 × 10ˆ6)
Solid-PRF (IntraSpin)	2700 rpm × 12 min (~700g)	5	20.7	33.7	2075	80.7	1.7	60.7
Solid-PRF (process)	1300 rpm × 8 min (~200g)	4.5	9.3	15.2	2,272.5	88.4	0.63	22.5
Solid-PRF (horizontal)	700g × 8	4.2	32.7	53.3	2,562	99.7	2.436	87.0
Liquid-PRF (IntraSpin)	2700 rpm × 3 min (~700g)	3.7	12.3	20.1	2020.2	78.6	1.073	38.3
Liquid-PRF (process)	800 rpm × 3 min (~60g)	1.3	9.8	16.0	690.3	26.9	0.884	31.6
Liquid-PRF (horizontal)	200g × 8	2	21.8	35.7	1,282	49.9	1.92	68.6

Abbreviation: PRF, platelet rich fibrin.

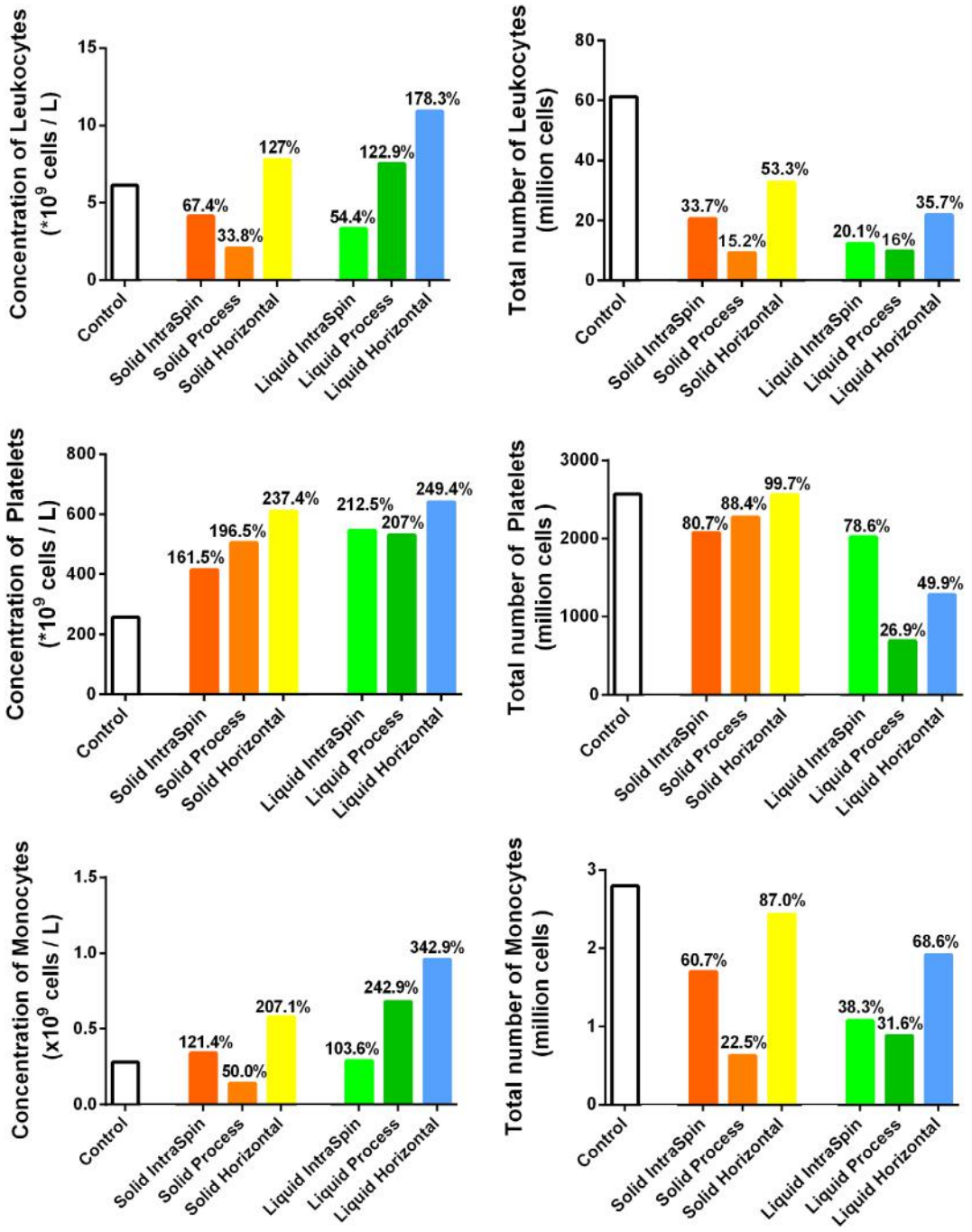

FIGURE 10 Graphical representation of the concentrations (left side) as well as total numbers (right side) of leukocytes, platelets and monocytes observed utilizing the various solid-PRF and liquid-PRF protocols. The percentage increase/decrease over baseline are marked accordingly (percentage of control whole blood concentrate and percentage of total cell yield). Notice that horizontal centrifugation produced the highest concentration and total number of cells for both solid-based and liquid-based protocols. PRF, platelet rich fibrin

to either the L-PRF or A-PRF protocols. Platelets concentrations were also increased between 25 and 50%. Furthermore, the liquid-PRF produced via horizontal centrifugation produced the highest concentration of platelets and leukocytes when compared to all other groups. These findings demonstrate convincingly that this new methodology to quantify cell types within PRF is effective to investigate PRF protocols and identify the precise location of cells following centrifugation. Though it is technically more demanding when compared to other methods owing to the greater number of samples analyzed, it provides an accurate observation of the precise position of the various cell types throughout each 1 mL layer found within the blood layers. Future studies utilizing this methodological protocol are now warranted to further optimize PRF protocols.

Two interesting phenomena were also noted in the present study. In each of the six tested participants, cell accumulation of leukocytes was routinely observed in layers 8–9 (and never in 10) in the A-PRF group. It remains intriguing to note that this trend was observed for

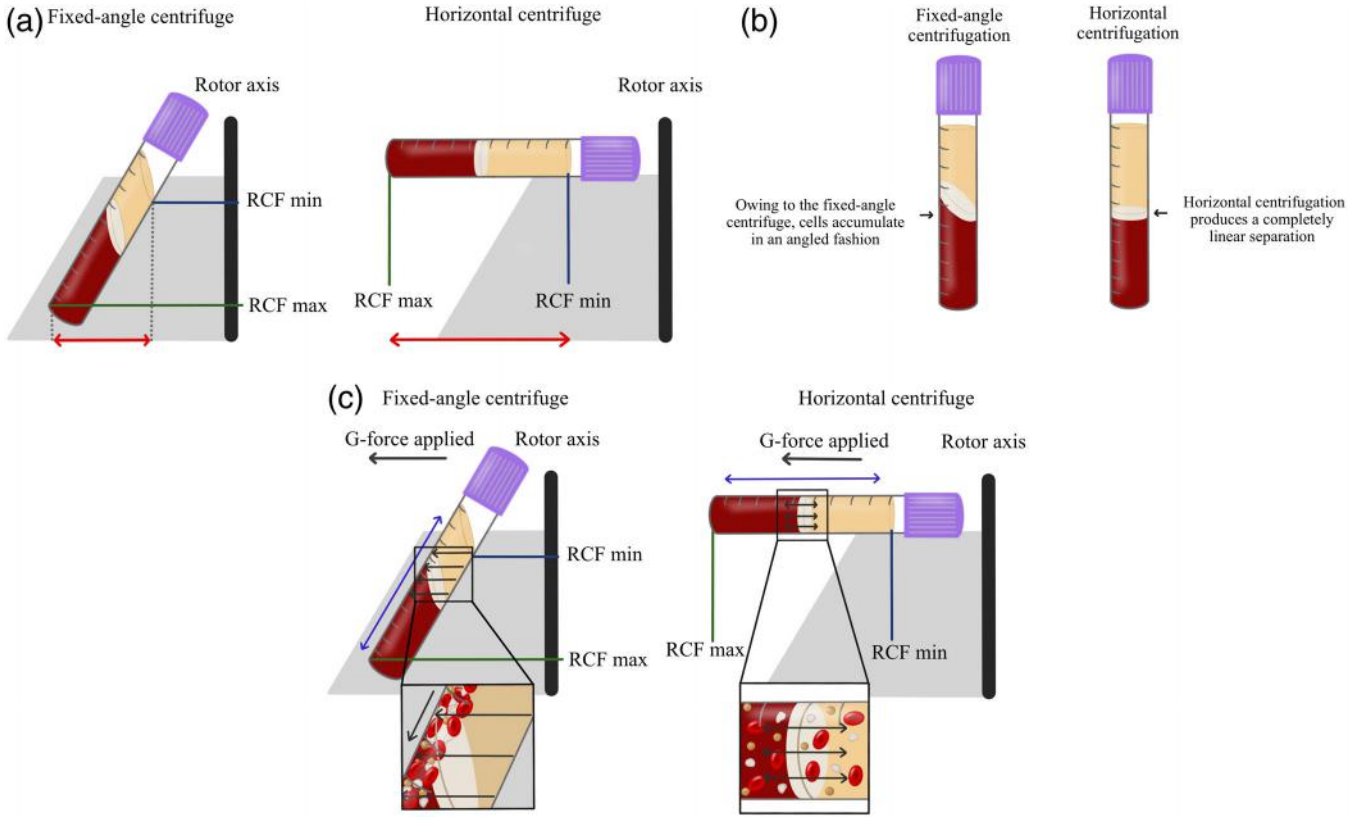

FIGURE 11 Illustrations comparing fixed-angle and horizontal centrifuges. (a) With fixed-angle centrifuges, a greater separation of blood layers based on density is achieved owing to the greater difference in RCF-min and RCF-max. (b) Following centrifugation on fixed-angle centrifuges, blood layers do not separate evenly and as a result, an angled blood separation is observed. In contrast, horizontal centrifugation produces an even separation. (c) Owing to the large RCF-values (~200–700g), cells are pushed toward the outside and downward. On a fixed-angle centrifuge, cells are pushed toward the back of centrifugation tubes and then downward/upward based on cell density. These g-forces produce additional shear stress on cells as they separate based on density along the back walls of centrifugation tubes. In contrast, horizontal centrifugation allows for the free mobility of cells to separate into their appropriate layers based on density allowing for more optimal cell separation as well as less trauma/shear stress on cells

all participants and only utilizing this protocol. As a result, we decided to compare both L-PRF and A-PRF protocols on three different fixed-angle centrifuges to verify the accuracy of this finding (data not shown). On each centrifugation device, this same trend was observed whereby leukocytes accumulated in the eighth and ninth blood layers. Future work to better understand this trend is ongoing.

The second interesting phenomenon observed was the large accumulation of platelets and leukocytes observed in layer 5 following L-PRF protocols. This single 1 mL layer was responsible for concentrating total platelet numbers at roughly 80%. In contrast, the 1 mL layer of i-PRF only accumulated a 25–30% platelet yield in a similar 1 mL volume. It therefore becomes possible to better accumulate platelets and leukocytes in liquid injectable PRF formulations by selectively harvesting this specific 1 mL layer found utilizing protocols centrifuged at higher RCF values. Future research is also ongoing to further optimize liquid PRF protocols.

5 | CONCLUSION

The present study found that horizontal centrifugation produced both solid and liquid formulations with both higher concentrations and numbers of platelets and leukocytes when compared to fixed-angle centrifuges. It also revealed that currently utilized manufacturer's recommended protocols require further optimization. Future research remains warranted.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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