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Journal of Medical Research and Innovation

Original Article

A novel approach of harvesting concentrated plasma-rich fibrin (PRF) with increased platelet count

N. Ragunanthan[1], C Anchana Devi[2], V. Hemavani[3]

[1]Department of Orthopaedics, RathiMed Speciality Hospitals, [2]PG and Research, Department of Biotechnology, Women's Christian College (Affiliated to University of Madras), [3]Department of Fertility, RathiMed Speciality Hospital, Chennai, Tamil Nadu, India.

***Corresponding author:**
C Anchana Devi,
PG and Research, Department of Biotechnology, Women's Christian College (Affiliated to University of Madras),
Chennai - 600 006, Tamil Nadu, India.

dr.anchanababu@gmail.com

Received: 10 January 2022
Accepted: 05 October 2022
EPub Ahead of Print: 03 November 2022
Published: 12 December 2022

DOI
10.25259/JMRI_1_2022

Quick Response Code:

ABSTRACT

Objectives: Platelet-rich fibrin (PRF) is an autogenous biomaterial, considered as the second-generation platelet concentrates rich in blood cells and growth factors entrapped in the fibrin matrix, which makes it as an ideal material with wound healing abilities. Studies have reported high harvest of cells using anticoagulants but the present study employs two different protocols to efficiently separate the platelet-rich layer at low centrifugal forces without the use of anticoagulants.

Material and Methods: Blood samples were collected with the consent of 20 volunteer donors. Ten blood samples were tested in each of the protocols studied, that is, protocol 1–200 g for 8 min (higher rpm and time) and protocol 2–60 g for 5 min (lower rpm and time). From the 12 ml of blood samples collected, 2 ml of blood was utilized for whole blood study; the remaining 10 ml was transferred into falcon tubes subjected to different rpm.

Results: The present study employs a novel method to investigate segregation of cell types following low-grade centrifugation. One milliliter sequential pipetting technique was used to analyze number of leukocytes and platelets following centrifugation at two different g-forces. The protocols followed in the present study had 2–3-fold increase leukocytes concentration and 10–12-fold increases in platelet concentrations in the layers than the whole blood sample without the use of anticoagulants.

Conclusion: The study concludes that protocol 1 was more efficient in harvesting platelets with less concentration of leukocytes, which is considered more suitable for various medical applications.

Keywords: Plasma-rich fibrin, Low-speed centrifugation, Platelet count, Leukocyte count

INTRODUCTION

Platelet concentrates have widely been used as a therapeutic option in the field of medicine such as dentistry, dermatology, orthopedics, and infertility.[1,2] Platelet-rich plasma (PRP) has been the standard preparation used extensively. PRP has got high to very high concentration of platelets but with less amount of natural fibrinogen. Since the use of platelet concentrate preparations in clinical practice, various protocols of platelet preparations have been described. Despite the success rate of PRP, several concerns have been raised regarding the effects of anticoagulants being used and their negative impact on tissue regeneration.[3-6] Platelet-rich fibrin (PRF) was, therefore, developed to reduce the use of anticoagulants. The preparation consisted of PRF clot. It was particularly useful in periodontology as the clot was applied like a membrane in the defect area.[7-11] In other medical fields, PRF has been utilized for the successful management of hard to heal and chronic leg ulcers.[12] PRP concentrate has to be converted to

a gel form by adding additional substances such as calcium chloride or thrombin but PRF clot is devoid of chemical substances, has natural fibrinogen, and easy to prepare by single-step centrifugation. Moreover, Kobayashi *et al*. have shown that growth factors are released over a period of time up to 10 days and total quantity of release was also higher than PRP.[13] Since PRF clot cannot be injected which is a disadvantage when compared to PRP, Mourao *et al*., in 2006, introduced injectable PRF.[14] It is available as liquid form though retaining all the advantages of PRF. Various protocols in preparing liquid PRF have been described in the literature.[15-18] It is pertinent to know much concentration and quantity of platelets can be obtained and how leukocytes are concentrated in a particular preparation.

Three different protocols are used to analyze leukocyte and platelet concentration and harvesting.[18] Two of the protocols used commercially available dedicated machines for PRF preparation with fixed-angle rotors. Third one utilized horizontal centrifuge machine as it has a better ability to separate cell types based on density due to its greater difference in relative centrifugal force (RCF) values between the RCF-min and RCF-max.[19] The first protocol utilized 700 g for 3 min, the second protocol used 60 g for 3 min, and finally, the third protocol used 200 g for 8 min. There is a vivid analysis of PRF preparation by sequential pipetting method. However, only one sample was analyzed in each group and only one horizontal method was used for a particular protocol. This study also included PRF clot preparations as well [Figure 1].

Based on the previous studies and reviews, one protocol with higher g-force and another with lower g-force were selected for the present study to understand the following questions:

1. Whether one could obtain consistent and comparable platelet concentration and quantity in liquid PRF using a conventional centrifuge machine
2. What concentration of platelets and leukocytes could be harvested within the supernatant in two different protocols?

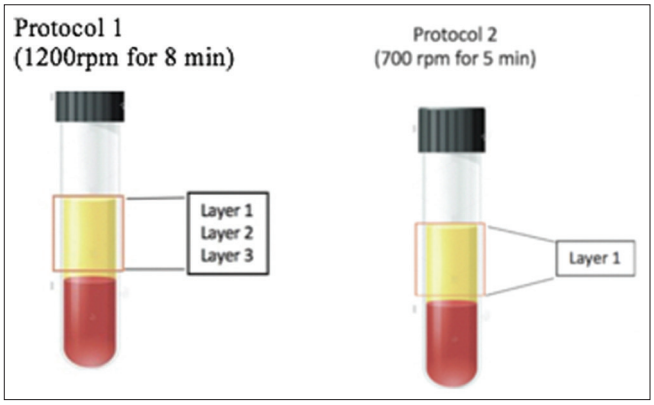

Figure 1: PRF preparation protocol 1 and protocol 2.

3. Which protocol gives better outcome in terms of platelet harvest?

MATERIAL AND METHODS

Preparation of PRF

Blood samples were collected with the consent of 20 volunteer donors. All the processes performed in this study involving human participants were performed in accordance with the ethical standards. The factors that affect fibrin formation and structures include genetic factors, acquired factors (such as abnormal concentration of thrombin and factor XIII in the plasma, blood flow, platelet activation, oxidative stress, hyperglycemia, hyperhomocysteinemia, medication, and cigarette smoking), and other parameters (concentration of chloride and calcium ions).[20] All patients with any of the above conditions were excluded from the study. All the included patients were systemically healthy, non-smoking, and were not under any medications.

A commercial laboratory centrifuge was utilized for the present study with the following features:

- Maximum speed of 4000 rpm
- Speed and time with incremental control
- Brushless inductor motor for smooth acceleration and braking

Each of the 20 volunteers donated 12 ml of blood. It was collected in a falcon tube since it is a non-toxic and non-pyrogenic tube and has a lesser influence on the cells and clotting mechanism. Ten blood samples were tested in each of the protocols studied, that is, protocol 1: 200 × g-force for 8 min (higher g-force and time) and protocol 2: 60 × g-force for 5 min (lower g-force and time). From the 12 ml of blood samples collected, 2 ml of blood was utilized for whole blood study; the remaining 10 ml was transferred into the falcon tubes and placed in the centrifuge immediately. The clinical success of the PRF protocol is dependent on a quick collection of blood and its transfer to the centrifuge machine. The tubes should always be balanced by opposing tube to equilibrate the centrifugation forces and to prevent vibrations during the process. After the spin, the plasma layer was aspirated immediately and sequentially from top in 1ml aliquots until red blood cell zone is reached. Sequential aspirations were transferred separately to fresh cuvettes for analysis of platelet and leukocytes counts. It was then compared with the same person whole blood platelet and leukocyte counts [Figure 2].

RESULTS

Normal values of complete blood count of whole blood are tabulated below [Table 1].

In protocol 1, after centrifugation at 200 × g for 8 min, an average 3 ml of plasma was obtained. Hence, we are able to

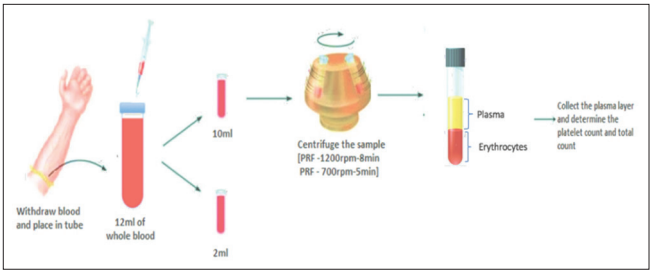

Figure 2: Clinical preparation of platelet-rich fibrin.

sequentially aspirate three layers of plasma (1 ml each). In whole blood, the total count of leukocytes was ranging from 5700 to 8100 cells/µl. In layer 1, the total leukocyte count was ranging from 400 to 5400 cells/µL and, in layer 2, there was a 2-fold increase in cell concentration ranging from 1000 to 10,500 cells/µL. Layer 3 had the maximum concentration of cells ranging 1800–24,000 cells/µL [Figure 3a]. Similarly, platelet concentration was compared between whole blood and the resultant plasma aspirations. The platelet concentration in whole blood was between 207,000 and 372,000 platelets/µL. The platelet concentration in layer 1 was between 409,000 and 884,000 platelets/µL. In layer 2, it varied between 430,000 and 961,000 platelets/µL, and in layer 3, it was in the range between 362,000 and 902,000 platelets/µL. It was inferred from the above figures that platelet concentration was higher in layers 2 and 3 [Figure 3b].

In protocol 2, after centrifugation at 60 × g for 5 min more or less, only one layer (around 1.5 ml) was obtained in all the samples. In whole blood sample, leukocyte count was observed to vary between 2800 and 13,500 cells/µL and platelet counts varied between 157,000 and 377,000 platelets/µL. In layer 1, the leukocyte count was observed to vary between 7100 and 16,800 cells/µL and platelet counts varied between 412,000 and 1,350,000 platelets/µL [Figure 4a and b].

In protocol 1 [Figure 5a and b], layers 1, 2, and 3 were rich in platelets that could be preferentially collected, directly above the red blood cell layer. There was a difference in leukocytes concentration between layers. Layers 1 and 2 had lower number of leukocytes compared to layer 3, whereas the platelet harvest in 1, 2, and 3 layers was almost similar. The standard deviation of the layers 1, 2, and 3 is ± 164,740.23, ± 172,848.8, and ± 1,651,110.8, respectively, and the standard mean error (SEM) was found to be ± 52,095.4, ± 54,659.6, and ± 52,212.6 which reflects that the harvest of platelets was more or less equal in all the layers without much deviation. In protocol 2, layer 1 the standard deviation was found to be ± 278,730 and SEM was ± 88,142.

The total platelets harvested in a preparation have been accepted as one of the major indices for ensuring the quality of platelet concentrates.[21] The harvest of platelets was higher in both the protocols when compared to whole blood

Table 1: Normal values of Complete blood count.

Components	Values and Ranges
White blood cell count (WBC)	4300-1080 cells per cubic millimeter (cmm)
White blood cell (WBC) differential count	The cells in a differential count are granulocytes, lymphocytes, monocytes, eosinophil, basophils
Red cell count (RBC)	4.2-5.9 million cells /cmm
Platelet count	150,000 to 400,000 per cmm

[Table 2]. Compared to protocol 2, protocol 1 has shown better harvest of total platelets. We could infer that protocol 1 is more efficient, based on application purpose due to its higher harvest of platelets. In protocol 1, 3 ml of plasma was obtained in six samples and 4 ml of plasma was obtained in the remaining samples. On an average, we were able to get 3.4 ml of plasma from 10 ml of sample using protocol 1. In protocol 1, on an average, we were able to harvest 73.5% (57–94%) of total platelets available in 10 ml of whole blood sample. In protocol 2, 1 ml of plasma was obtained in nine samples and 1.6 ml of plasma was obtained in one sample. On an average, we were able to get 1.06 ml of plasma from 10 ml of sample using protocol 2. In protocol 2, on an average, we were able to harvest only 25.9% (17–38%) of platelets [Figure 6].

From [Table 3], it is observed that there is a significant statistical difference (with confidence level of 95%) in harvesting of leukocytes among different layers of sample using protocol 1. Further mean scores reveal that the use of layer 1 seems to have low number of leukocytes. Whereas, the platelet count between and within the layers did not have a significant F value −3.798 that indicates that there is no significant confidence level (*P* value) reflected among the layers and within the layers, which infers that the harvest of the platelets in different layers is almost same using protocol 1.

DISCUSSION

Platelet concentrates have been extensively used in various clinical specialties and PRF preparations are gaining momentum in recent years outside the domain of dentistry. Although different protocols have been described for PRF preparations, there is no investigation which suggests a particular protocol yields comparable if not superior platelet concentration and harvest. The efficiency of different protocols in liquid PRF preparation was analyzed,[18] but they have compared using different machines and only one sample was used for each protocol. Hence, we have used single conventional centrifuge machine with horizontal bucket to analyze two different protocols described in the literature.

A sequential pipetting methodology was used to accurately quantify the effect of centrifugation protocols on the

Figure 3: (a) Leukocyte count in different layers of the samples (1–10) using protocol 1. (b) Platelet count in different layers of the samples (1–10) using protocol 1.

separation of cell layers.[12] This method was developed to accurately identify the location of cells following various centrifugation protocols. A control group to compare and evaluate the number of cells in the form of whole blood was also carried out simultaneously for protocols 1 and 2 [Figure 7].

In protocol 1, which used 200g force for 8min gave higher plasma volume(average of 3.4 ml) and had high platelet concentration (220%) which was evenly distributed throughout the plasma layers. In protocol 2 which involved lower g-force (60 g), the obtained plasma volume was much lower (1.2 ml), though the concentration

Table 2: Leukocytes and platelets harvest – protocols 1 and 2.

Sample no	Final volume of plasma obtained from 10 ml of whole blood	% of platelet harvested from 10 ml of. sample	Final volume of plasma obtained from 10 ml of whole blood	% of platelet harvested from 10 ml of sample
1	3 ml	57%	1 ml	22%
2	3 ml	83%	1 ml	28%
3	4 ml	70%	1.6 ml	31%
4	4 ml	73%	1 ml	38%
5	4 ml	78%	1 ml	17%
6	4 ml	94%	1 ml	20%
7	3 ml	93%	1 ml	25%
8	3 ml	65%	1 ml	23%
9	3 ml	54%	1 ml	25%
10.	3 ml	68%	1 ml	30%
Avg	3.4ml	73.5%	1.06 ml	25.9%

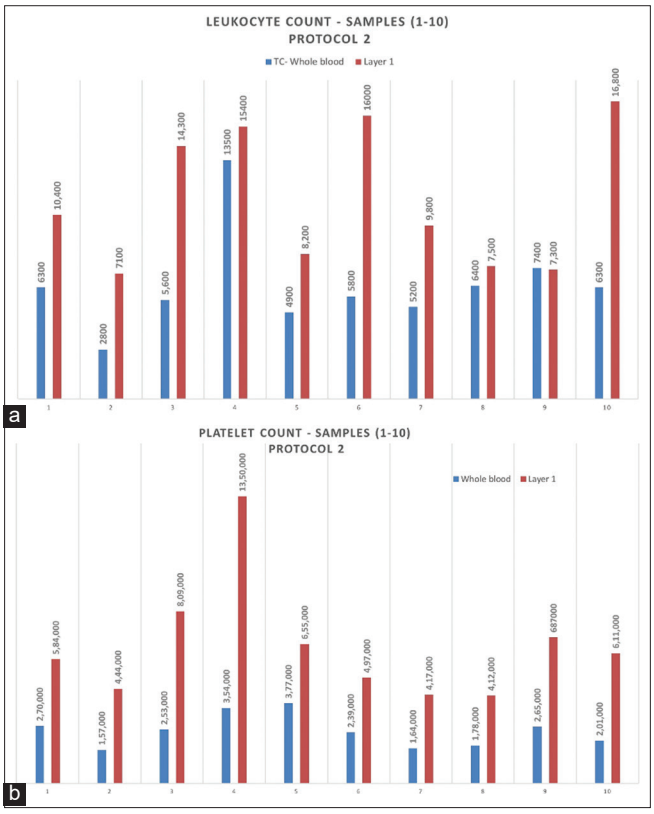

Figure 4: (a) Leukocyte count indifferent layers of the samples (1–10) using protocol 2. (b) Platelet count in different layers of the samples (1–10) using protocol 2.

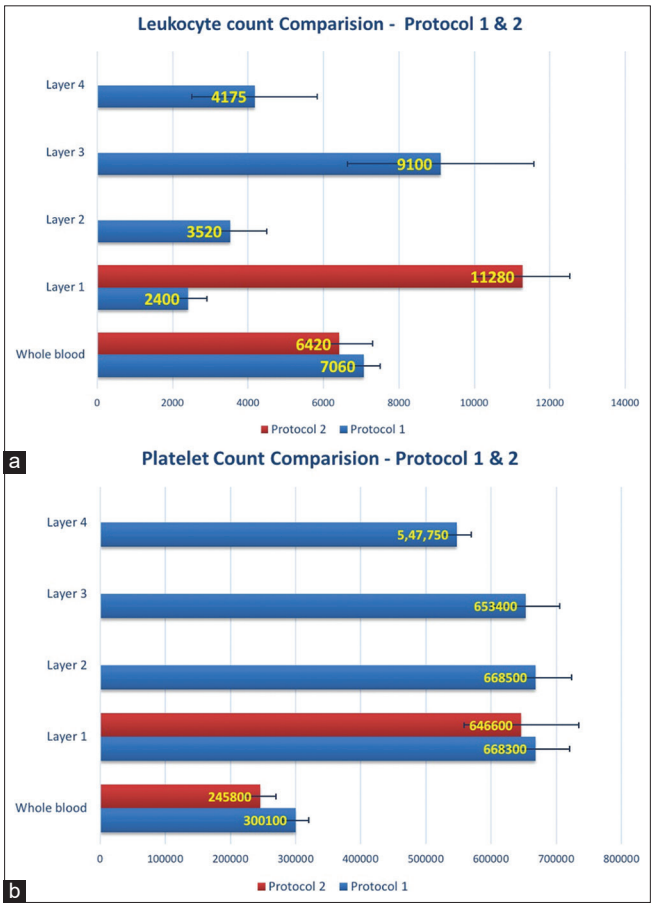

Figure 5: (a) Comparison of leukocyte count – protocols 1and 2. (b) Comparison of platelet count – protocols 1 and 2.

(265%) was little more efficient than protocol 1. However, there was huge difference in total platelet harvest between the protocols. The average platelet harvest in protocol 1 is around 73.5% (57–94) and in protocol 2 is around 26% (17–38). Similar finding was found in Miron *et al.* study in 2019. In Miron's study, they compared three different protocols of liquid PRF preparations and found that low RCF centrifuge protocol (60 g) has not yielded adequate platelet harvest on contrary to the previous literature and manufacturer's (PRF process) recommendations. These findings carry important significance as higher platelet harvest with higher plasma volume has more clinical value and higher platelet concentration alone does not carry significance.

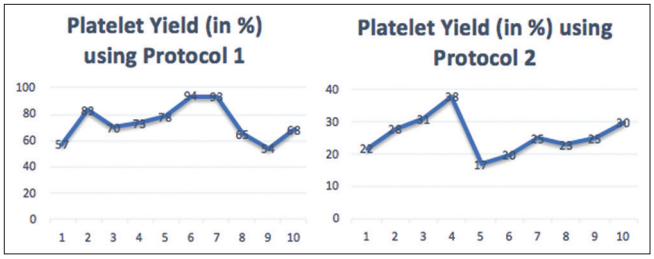

Figure 6: Platelet yields (in %) using protocols 1 and 2.

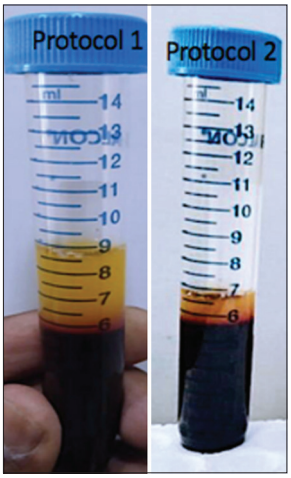

Figure 7: Plasma separation using protocols 1 and 2.

Horizontal tube centrifugation allows greater separation of cell layers due to greater differential between the minimum and maximum radius found within a centrifugation tube. Furthermore, centrifugal forces in fixed-angle devices cause more damage to the cells. In fixed-angle devices, cells are pushed against the wall of the test tube and in this process larger RBCs trap platelets and drag them into red zone. In horizontal centrifuges, there is no such phenomenon; hence, there is clear separation of cells based on their mass[18] [Figure 8].

It is also imperative to compare the platelet harvest of liquid PRF prepared with horizontal centrifuge against PRP preparations as both represent liquid forms. Fitzpatrick *et al.*, in 2017, compared platelet concentration and harvest among four different commercial kits. Excluding ACP kit other three kits, namely, GPS, Smart Prep, and Magellan, had platelet concentration from 3.5 to 4.5 times the control values. This level of concentration looks impressive.[22] However, they have not looked into total platelet harvest from a given volume. Based on the data presented in the article and by reverse calculation, we are able to arrive total platelet harvest from a given volume of say 10 ml. GPS system gave 1.25 ml of plasma achieving 44.8% platelet harvest. In both Smart Prep and Magellan kits, 1.25 ml of plasma and 57% harvest were achieved. One could easily infer that though platelet

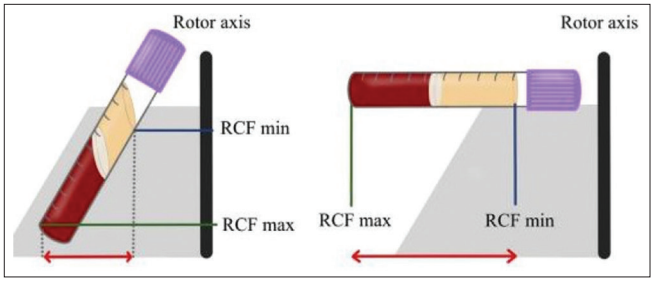

Figure 8: Horizontal centrifugation.

Table 3: One-way analysis of variance among different layers with respect to leukocyte and platelet counts in protocol 1.

Parameters	Mean	Df	Statistical inference
Leukocyte	Layer 1 – 2400		
Count	Layer 2 – 3590		F=3.798
Between layers	Layer 3 – 9100	2	P< 0.05
Within layers			Significant
Platelet	Layer 1 – 668,300		
Count	Layer 2 – 668,500		F= 0.9326
Between layers	Layer 3 – 653,400	3	*P*-value
Within layers	Layer 4 – 547,750		Not significant

concentration was much higher in commercial PRP kits when compared to PRF preparation by horizontal centrifuge method (2.2 times) since the plasma volume (protocol 1) is higher (3.2 ml), platelet harvest is much higher with this technique (73.5%).

In protocol 1, WBCs are 70% concentrated when compared to control values, and in protocol 2, 175% of WBCs are concentrated. This finding contrasts with Miron study which had 178% concentration in horizontal centrifuge method (Miron *et al.*, 2019). PRP commercial kits concentrated WBCs by more than 250%. In terms of harvest, nearly 65% of WBCs are concentrated in protocol 1 compared to 35–50% harvest in commercial L-PRP kits. Concentration and harvest of WBC have two important implications. One is its antimicrobial activity against both Gram-positive and -negative bacteria.[23] Other implication is WBCs release catabolic cytokines which might be harmful to cartilage when injected into synovial joint. However, these observations are made *in vitro* studies.[24] However, clinical studies to demonstrate the catabolic effect of joint cartilage are sparse. Filardo *et al.* showed that leukocyte-rich (LR) PRP injections did not have any long-term deleterious effect on joints though these injections elicited localized pain and swelling within 6 h of injection.[25] Masahiko Kenmachi, in 2020, analyzed outcome of LR-PRP in 50 knees and followed for 6 months.[26] It was found to give favorable outcomes in more than 74% of the knees. Mariani *et al.*, in 2016, published extensive *in vivo* analysis of injection of LR PRP in knee joints.[27] They analyzed the levels of pro-

inflammatory and anti-inflammatory cytokines post-LR-PRP and HA injections. There was no specific up regulation of inflammatory mediators at 7 days of follow-up. In fact, there was downregulation of IL 8 a pro-inflammatory marker in LR-PRP injection group when compared to HA group. Hence, the speculation of damage to joint cartilage by high leukocyte count in platelet preparations based on *in vitro* studies may not translate to same effect clinically.

Surprising finding in our analysis is that top 1 and 2 layers had lower concentration of leukocytes (<50%) when compared to control whole blood values. This effect was observed consistently across all the samples in protocol 1. Until convincing evidence is available that LR platelet preparations do not cause pro-inflammatory damage to critical structures, one can selectively aspirate the top 2 ml of plasma which does not have high concentration of leukocytes.

CONCLUSION

The present study describes a novel approach for investigating the cell concentration and harvest directly above the red blood cell layer. This study shows that higher concentration of platelets can be achieved using conventional horizontal centrifuges. Low g-force (200 g) protocol yields better harvest of platelets compared to lower g (60 g) force protocol. It is also easier to aspirate plasma with high platelet concentration in the first protocol as there was uniform concentration of platelets in all the plasma layers above red zone, whereas one must selectively aspirate the buffy coat in second protocol. First, protocol (75%) scores better in terms of total platelet harvest when compared to commercial PRP kits (around 50%) though commercial PRP kits could concentrate more than 3 times. This difference could be advantageous in areas where large numbers of platelets are needed with minimal amount of autologous blood harvest. Furthermore, PRF preparations have higher content of natural fibrin and have no added chemicals compared to PRP preparations. This may lead to more natural interaction at host repair site with sustained growth factor release.

Differential concentration of leukocyte content allows clinician to aspirate selectively layers with lesser concentration. Hence, until more evidence is available that high leukocyte count is not harmful to cartilage this method of PRF preparation could be used in the treatment of cartilage damage and osteoarthritis.

Benchtop horizontal centrifuge can reliably give a working type liquid PRF product which can be used in wide variety of clinical applications. Further comparative pre-clinical and clinical research studies are required to strengthen this concept of PRF preparation.

Declaration of patient consent

Patient's consent not required as patient's identity is not disclosed or compromised.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Ragunanthan N, Anchana Devi C, Hemavani V. A novel approach of harvesting concentrated plasma-rich fibrin (PRF) with increased platelet count. J Med Res Innov 2022;6:11-8.