

Standardization of Platelet-Rich Plasma Preparation: Importance of centrifugal force and time.

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ABSTRACT: Backgrounds- Platelets are rich in growth factors and cytokines which play a crucial role in wounds healing and tissue regenerative process. The wide variation in reported protocol for PRP preparation leads to variable compositions and biological response. Detailed, precise and stepwise description for PRP preparation with composition is required.

Aims: Analyze stepwise recovery efficiency of platelets and WBCs in PRP on different centrifugal force and time at 22°C.

Materials and method: 5 mL whole blood from 60 participant's Contains CPDA-1 anticoagulant was collected into each 6 subgroup test tube. For 1st spin (PRP-1): group-A (30 samples), centrifuged at 700g for- 2 min (A-I), 3 min (A-II), 4 min (A-III), 5 min (A-IV), 6 min (A-V), 7 min (A-VI) and Group-B (30 Samples), at 200g for 8 min (B-I), 9 min (B-II), 10 min (B-III), 11 min (B-IV), 12 min (B-V) and 13 min (B-VI). For 2nd spin (PRP-2): Group-A (30 samples), at 700g for 19 min (A-I), 17 min (A-II), 15 min (A-III), 13 min (A-IV), 11 min (A-V), 9 min (A-VI) and Group-B (30 Samples) at 1500g for- 2 min (B-I), 3 min (B-II), 4 min (B-III), 5 min (B-IV), 6 min (B-V) and 7 min (B-VI)

Results: after 1st spin (PRP-1) in group-A (700g), at 3 min platelet recovery was 88.61±8.59% with residual WBC of 3.10±2.22%. At 7 min platelet recovery was 78.01±9.90% with residual WBC of 0.93±0.45%. In group-B (200g), at 11 min platelet recovery was 84.78±8.5% with residual WBC of 1.31±3.07%. At 12 min platelet recovery was 84.63±7.1% with residual WBC of 0.90±0.47%. After 2nd spin (PRP-2 from PRP-1) in group-A (700g) at 15 min platelet recovery was 92.71±11.7% and on 1500g at 7 min platelet recovery was 96.84±7.77%.

Conclusion: Different composition of platelets and WBCs in PRP can be prepared by adjusting centrifugal force and time. Centrifugation at 22°C for 1st spin at 200g for 12 min and 2nd spin at 1500g for 6 min was optimal condition for PRP preparation.

Key Words: Platelet-rich plasma; Regeneration; Growth factors; Cytokine; standardization.

I. Introduction:

Platelet contains growth factors in their α -granules including; platelet-derived growth factor, transforming growth factor- β , platelet-derived epidermal growth factor, vascular endothelial growth factor, insulin-like growth factor-1, fibroblastic growth factor, and epidermal growth factor.[1] platelets also have an anti-inflammatory, analgesic [2-7] and antibiotic effect.[8] Platelets prevent acute blood loss by repairing vascular walls and adjacent tissues after injury. Platelets are activated by collagen after endothelial injury, they aggregate and secrete stored intercellular mediators and cytokines from their cytoplasmic pool and release their α -granule content. These secretions are very high during first hour and continue for at least another one week with their synthesis,[9] The >800 types of proteins are secreted into surrounding media,[10] induce signal to different cell types: myocytes,[11] tendon cells,[11-14] mesenchymal stem cells from different origins,[15-16] chondrocytes,[17] osteoblasts,[18] fibroblasts[19] and endothelial cells.[20] These signaling cytokines and growth factors play an important role in regeneration and healing process of soft and hard tissues by regulating inflammation, stimulating collagen synthesis, cell proliferation, cell migration, angiogenesis and synthesis together with the remodeling of newly formed tissues.[21 -24]

Platelet rich plasma (PRP) is an autologous preparation that concentrates platelets into small volume of plasma.[25-26] Numerous studies have reported the clinical application of PRP and their promising results in wound healing and regenerative process in different fields of medical science.[22, 27-46] However, controversies were found regarding the stem cell differentiation capacity, some studies reported PRP

avored the osteogenic differentiation[15] while others demonstrated a chondrogenic compromise.[16-47] These variations could be due to different protocols used for PRP preparations and their composition, which can modify its growth factor content.[26,48] Studies have reported that cooling retards platelet activation,[49] and growth factors decrease after use of high centrifugal force during PRP preparation. Some authors reported, WBCs provide natural protection against infections and allergic responses.[50-51] Others correlate presence of high WBC concentration in PRP with increasing inflammation and reducing tissue regeneration,[52] due to their catabolic cytokine concentration (matrix metalloproteinase-9 and IL-1β),[53] catabolic gene expression,[53-54] and release of toxic reactive oxygen species,[52] destroying surrounding tissue, even if tissue is not injured.[55-56] According to a previous study, PRP prepared should have 3 to 4 fold more platelets than whole blood for therapeutic benefits. The study reported lower concentrations are unreliable in enhancing wound healing and higher concentration may not show further benefits.[57] At the moment, there is need of a detailed, precise and stepwise description (centrifugation force, time, temperature, platelet and WBC concentration with anticoagulants used) of the PRP preparation protocol.[58-65]

The purpose of this study is to analyze stepwise platelets recovery and WBCs in PRP, at different centrifugal accelerations and time, on standard temperature 22°C (20°C -24°C) to develop a protocol to obtain PRP with standard quality and higher recovery of platelets, by using basics and fundamental principles involved in blood component (PRP) preparation.4

2. Materials and method:

2.1. Blood collection: After the approval of Hospital Local Ethics Committee, 60 healthy adult Volunteer participants between 18-60 years of age were enrolled to the study. All participants gave informed consent. The participants were divided into two Groups namely- Group-A (30 donor) and Group-B (30 donor) and both groups sample were divided into 6 subgroups (group-A, 30 donors into subgroups- A-I, A-II, A-III, A-IV, A-V, A-VI and group-B ,30 donors into subgroups- B-I, B-II, B-III, B-IV, B-V, B-VI). For blood collection from each participant, 3.92 mL of anticoagulant (0.14/mL blood) CPDA-1was collected in 50 ml syringe. Needle was replaced with 18-guage needle and 28 mL of each participants blood was collected in the syringe (total volume, anticoagulant + blood=31.92 mL) from antecubital region within 2-6 minutes. 5 mL of whole blood was transferred into each test tube of 6 separate subgroups test tube, for PRP preparation. Remaining 1.9 mL blood of each participant was used for baseline blood cell count. Blood cell count was performed with cell counting equipment (Sysmex KX-21, Sysmex corporation Japan).

2.2. PRP Preparation: All centrifugal process was done in a Refrigerated centrifuge model (ROTO SILENTA 630 RS, Hettich, Instruments LP. Germany). All samples were centrifuged in two steps (1st and 2nd spin) with the RCF and time as shown in figure-1 and Brief protocol described in figure-2.

		1 st spin at 22°C	RCF	1 st spin Centrifugal Time in min.	2 nd spin at 22°C	RCF	2 nd spin Centrifugal Time in min.
Group-A 30 participants	A-I	700g		2	700g		19
	A-II			3			17
	A-III			4			15
	A-IV			5			13
	A-V			6			11
	A-VI			7			9
Group-B 30 participants	B-I	200g		8	1500g		2
	B-II			9			3
	B-III			10			4
	B-IV			11			5
	B-V			12			6
	B-VI			13			7

Figure-1: RCF and time for PRP preparation for 1st spin (PRP-1) and 2nd spin (PRP-2)

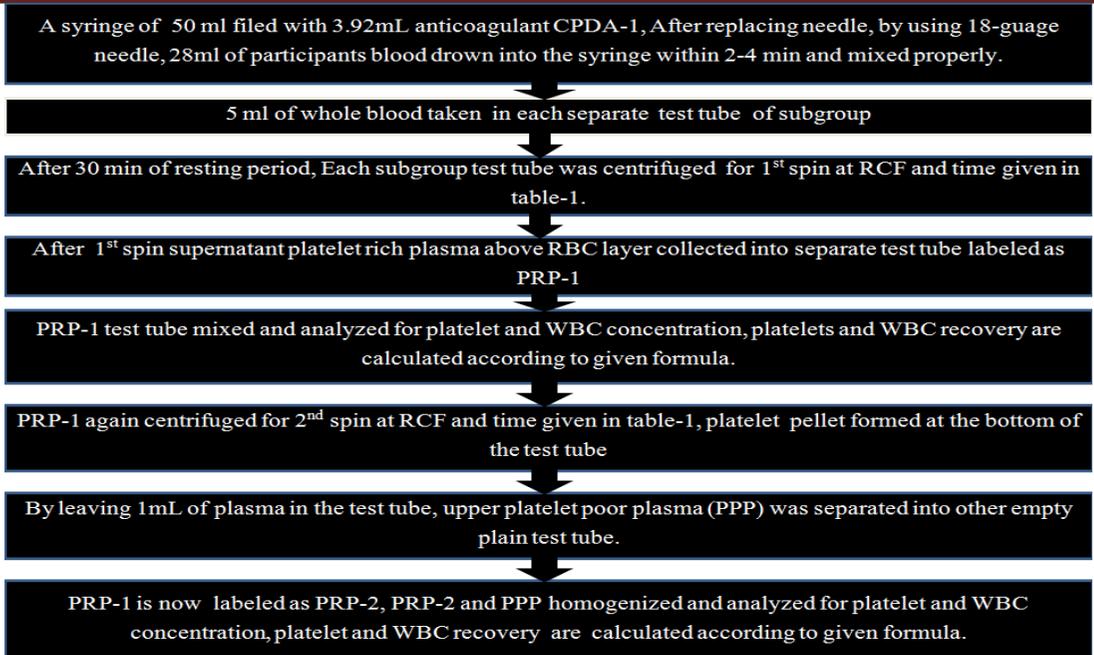


Figure-2: Flow chart, describing brief protocol of PRP preparation.

2.2.1. First Spin: After the formation of three layers (a bottom layer composed of RBCs an upper layer composed of plasma, platelets and some WBCs and an intermediate layer or Buffy coat composed mostly of WBCs), the upper layer was collected with a micro pipette. This collection was performed carefully to avoid disturbing the bottom layer of RBC and the Buffy coat layer. Depending on the centrifugal force of the spin, the collected volume ranged from 1.89-2.34 mL. The collected sample was then transferred to an empty test tube and homogenized, called PRP-1. After the sample was adequately mixed, blood cell count was performed with cell counting equipment (Sysmex KX-21). Percentage platelet yield in PRP-1 and PRP-2 compared to baseline was calculated using the following formula/equations:

$$1. \text{ Percentage Platelet recovery in PRP1} = \frac{\text{Total Platelets in PRP 1}}{\text{Total Platelets in Baseline}} \times 100$$

$$2. \text{ Percentage residual WBC in PRP1} = \frac{\text{Total WBCs in PRP 1}}{\text{Total WBCs in Baseline}} \times 100$$

2.2.2. Second Spin: Platelets pellet is formed at the bottom of the test tube. By leaving 1 mL of plasma, the upper platelet poor plasma (PPP) was separated into the other empty plane test tube, by the help of micropipette. PRP-1 is now named as PRP-2 (platelet concentrate). PRP-2 and PPP was homogenized and analyzed for platelets and WBCs count. Percentage platelet yield and residual WBCs in PRP-2 compared to baseline and PRP-1 were calculated using the following equations:

$$3. \text{ Percentage Platelet recovery in PRP2 from baseline} = \frac{\text{Total Platelets in PRP 2}}{\text{Total Platelets in Baseline}} \times 100$$

$$4. \text{ Percentage Platelet recovery in PRP-2 from PRP1} = \frac{\text{Total Platelets in PRP 2}}{\text{Total Platelets in PRP 1}} \times 100$$

$$5. \text{ Percentage residual WBCs in PRP2 from baseline} = \frac{\text{Total WBCs in PRP 2}}{\text{Total WBCs in Baseline}} \times 100$$

$$6. \text{ Percentage residual WBCs in PRP2 from PRP1} = \frac{\text{Total WBCs in PRP 2}}{\text{Total WBCs in PRP 1}} \times 100$$

2.3. Statistical analysis: For statistical analysis data was entered into software Microsoft excel (sheet) and analysis was performed with SPSS Software (SPSS 13.0) for Windows (SPSS Inc., Chicago, IL, USA). Multiple groups comparison was assessed using ANOVA / Kruskal-Wallis H-test, inter subgroup comparison was assessed using t-test / Mann-Whitney U-test. A P. value of <0.05 was considered statistically significant for all statistical analysis.

3. Results and observations:

3.1. Composition after First spin:

After the 1st spin, the concentration of platelets and WBC in the separated upper layer (PRP-1) was measured.

3.1.1. For group-A:

After 1st spin of group-A sample (PRP-1, Table-1), the difference among subgroups- A-I to A-VI of mean platelet concentration, mean fold increase in platelet concentration, mean PRP-1 Volume, percentage platelet recovery was statistically significant (All $P < 0.05$, ANOVA).

Pair wise *t*-test (PRP-1, Table-1) was performed between these subgroups, the difference in mean fold increase in platelet concentration between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-III, II-IV, II-V, II-VI, III-V, III-VI, IV-VI, mean PRP-1 Volume between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI and mean percentage platelet recovery between pair wise subgroups- I-II, I-VI, II-V, II-VI, III-V, III-VI, IV-VI, was statistically significant.

The mean total platelet count (PRP-1, Table-1) was highest ($849.44 \pm 222.9 \times 10^6$) at 3 min with mean percentage platelet recovery $88.61 \pm 8.59\%$.

Table 1: Results of group-A samples after 1st Spin (Platelet and WBC concentration and recovery in PRP-1)

Subgroups	A-I (n=30)	A-II (n=30)	A-III (n=30)	A-IV (n=30)	A-V (n=30)	A-VI (n=30)
Mean PRP-1 volume in mL	1.890 ± 0.292	2.028 ± 0.296	2.146 ± 0.278	2.231 ± 0.269	2.328 ± 0.262	2.346 ± 0.241
Mean platelet concentration PRP-1 $\times 10^3 / \mu\text{L}$	431.60 ± 104.7	423.96 ± 110.2	394.93 ± 103.7	367.26 ± 105.9	338.70 ± 83.1	323.80 ± 94.4
Mean total platelet count in PRP-1 $\times 10^6$	805.81 ± 205.1	849.44 ± 222.9	838.91 ± 222.4	811.13 ± 229.2	780.66 ± 184.3	750.79 ± 211.1
Mean fold increase in platelet concentration PRP-1	2.26 ± 0.26	2.21 ± 0.27	2.06 ± 0.26	1.91 ± 0.31	1.77 ± 0.24	1.68 ± 0.29
Mean % platelet recovery in PRP-1	84.21 ± 7.36	88.61 ± 8.59	87.35 ± 7.82	84.17 ± 10.33	81.90 ± 6.70	78.01 ± 9.90
Mean WBC concentration PRP-1 $/ \mu\text{L}$	1183.33 ± 1108.3	593.33 ± 539.0	263.33 ± 247.0	176.67 ± 197.7	146.67 ± 116.7	133.33 ± 66.1
Mean total WBC count In PRP-1 $\times 10^6$	2.06 ± 1.80	1.09 ± 0.88	0.54 ± 0.45	0.38 ± 0.41	0.34 ± 0.31	0.31 ± 0.13
Mean % WBC concentration in PRP-1 from baseline	5.94 ± 5.28	3.10 ± 2.22	1.58 ± 1.30	1.13 ± 1.08	1.02 ± 0.87	0.93 ± 0.45

Mean baseline platelet concentration was $190.8 \pm 40.85 \times 10^3 / \mu\text{L}$ with mean WBC concentration $6.83 \pm 1.16 \times 10^3 / \mu\text{L}$ and total platelet count $954.0 \pm 204.2 \times 10^6$ with total WBC count 3.4 ± 0.58 in 5 mL of whole Blood.

After 1st spin of group-A sample (PRP-1, Table-1), the difference among subgroups- A-I to A-VI of mean WBC concentration, mean total WBC count, mean percentage residual WBC concentration in PRP-1, from baseline was statistically significant (All $P < 0.05$, Kruskal-Wallis H-test).

Pair wise Mann-Whitney U-test (PRP-1, Table-1) was performed between these subgroups, the difference in mean WBC concentration between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-III, II-IV, II-V, II-VI, III-IV, III-V, III-VI, mean total WBC count between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-III, II-IV, II-V, II-VI and mean percentage residual WBC concentration in PRP-1 from baseline between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-III, II-IV, II-V, II-VI, was statistically significant.

The percentage residual WBC concentration from baseline (PRP-1, Table-1) was lowest $0.93 \pm 0.45\%$ at 7 min.

3.1.2. For group-B:

After 1st spin of group-B sample (PRP-1, Table-2) the difference among subgroups- B-I to B-VI of mean fold increase in platelet concentration was statistically significant (All $P < 0.05$, ANOVA).

Pair wise *t*-test (PRP-1, Table-2) was performed between these groups, the difference in mean fold increase platelet concentration between pair wise subgroups- I-V, I-VI, II-IV, II-V and II-VI was statistically significant.

The mean total platelet count in PRP-1 (PRP-1, Table-2) was highest ($906.75 \pm 286.6 \times 10^6$) with mean percentage platelet recovery $84.78 \pm 8.5\%$ at 11 min.

Table-2: Results of group-B samples after 1st Spin (Platelet and WBC concentration and recovery in PRP-1)

Subgroups	B-I (n=30)	B-II (n=30)	B-III (n=30)	B-IV (n=30)	B-V (n=30)	B-VI (n=30)
Mean PRP-1 volume in mL	1.966 ± 0.37	1.970 ± 0.44	2.056 ± 0.38	2.130 ± 0.37	2.168 ± 0.39	2.161 ± 0.40
Mean platelet concentration PRP-1 $\times 10^3/\mu\text{L}$	456.17 ± 136.9	460.07 ± 142.6	443.53 ± 133.4	431.17 ± 131.9	423.03 ± 128.1	418.16 ± 129.1
Mean total platelet count in PRP-1 $\times 10^6$	890.20 ± 287.5	891.38 ± 297.8	902.11 ± 286.3	906.75 ± 286.5	903.39 ± 283.2	888.22 ± 279.4
Mean fold increase in platelet concentration PRP-1	2.14 ± 0.27	2.17 ± 0.26	2.09 ± 0.23	2.02 ± 0.25	1.99 ± 0.25	1.97 ± 0.27
Mean % platelet recovery in PRP-1	83.06 ± 12.1	83.40 ± 11.6	84.54 ± 8.8	84.78 ± 8.5	84.63 ± 7.1	83.31 ± 8.9
Mean WBC concentration PRP-1 $/\mu\text{L}$	543.33 ± 642.6	406.67 ± 437.0	323.33 ± 415.0	223.33 ± 209.6	136.67 ± 61.5	150.00 ± 97.4
Mean total WBC count in PRP-1 $\times 10^6$	1.03 ± 1.25	0.78 ± 0.87	0.65 ± 0.86	0.46 ± 0.46	0.29 ± 0.14	0.31 ± 0.22
Mean % WBC concentration in PRP-1 from baseline	2.82 ± 3.07	2.09 ± 1.94	1.73 ± 1.80	1.31 ± 1.00	0.90 ± 0.47	0.92 ± 0.49

Mean of baseline platelet concentration was $211.27 \pm 56.12 \times 10^3/\mu\text{L}$ with mean WBC concentration $6.83 \pm 1.44 \times 10^3/\mu\text{L}$ and total platelet count $1056.33 \pm 280.64 \times 10^6$ with total WBC count $3.41 \pm 0.72 \times 10^7$ in 5 mL whole Blood.

After 1st spin of group-B sample (PRP-1, Table-2), the difference among subgroups- B-I to B-VI of mean WBC concentration, mean total WBC count, mean percentage residual WBC concentration in PRP-1 from baseline was statistically significant (All $P < 0.05$, Kruskal-Wallis H-test).

Pair wise Mann-Whitney U-test (PRP-1, Table-2) was performed between these subgroups, the difference in mean WBC concentration between pair wise subgroups- I-IV, I-V, I-VI, II-IV, II-V, II-VI, mean total WBC count between pair wise subgroups- I-IV, I-V, I-VI, II-V, II-VI and mean percentage residual WBC concentration from baseline between pair wise subgroups- I-IV, I-V, I-VI, II-V, II-VI, was statistically significant.

The mean percentage residual WBC concentration from baseline (PRP-1, Table-2) was lowest ($0.90 \pm 0.47\%$) at 12 min.

3.2. Composition after Second Spin:

3.2.1. For group-A:

After 2nd spin of group-A samples (PRP-2, Table-3), the difference among subgroups- A-I to A-VI of mean platelet concentration, mean fold increase in platelet concentration in PRP-2 from PRP-1, mean fold increase in platelet concentration in PRP-2 from baseline, mean total platelet count in PRP-2, percentage platelet recovery PRP-2 from PRP-1, percentage platelet recovery PRP-2 from baseline and mean residual platelet in PPP was statistically significant (All $P < 0.05$, ANOVA).

Pair wise *t*-test (PRP-2, Table-3) was performed between these subgroups, the difference in mean platelet concentration between pair wise subgroups- I-VI, II-V, II-VI, III-VI, IV-VI, mean fold increase in platelet concentration in PRP-2 from PRP-1 between pair wise subgroups- I-III, I-IV, I-V, I-VI, mean fold increase of platelet concentration in PRP-2 from baseline between pair wise subgroups- I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI, IV-VI, mean total Platelet count in PRP-2 between pair wise subgroups- I-VI, II-V, II-VI, III-VI, mean percentage platelet recovery in PRP-2 from PRP-1 between pair wise subgroups- I-VI, II-VI, III-VI, mean percentage platelet recovery in PRP-2 from baseline between pair wise subgroups - I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI, IV-VI and mean residual Platelet in PPP between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI, IV-V, IV-VI, was statistically significant.

The mean percentage platelet recovery in PRP-2 from PRP-1 (PRP-2, Table-3) was highest ($92.71 \pm 11.7\%$) with mean platelet concentration $771.17 \pm 199.4 \times 10^3 / \mu\text{L}$ at 15 min. however, mean platelet concentration was highest ($773.47 \pm 196.6 \times 10^3 / \mu\text{L}$) with mean fold increase in platelet concentration from baseline 4.04 ± 0.43 -fold and mean percentage platelet recovery from baseline $80.95 \pm 8.7\%$ at 17 min.

After 2nd spin of group-A sample (PRP-2, Table-3), the difference among subgroups- A-I to A-VI of mean WBC concentration, mean total WBC count and mean percentage residual WBC concentration in PRP- 2 from baseline, was statistically significant (All $P < 0.05$, Kruskal-Wallis H-test).

Pair wise Mann-Whitney U-test (PRP-2, Table-3) was performed between these subgroups, the difference in mean WBC concentration between pair wise subgroups- I-II, I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-IV, III-V, III-VI, mean total WBC count between pair wise subgroups- I-II, I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-IV, II- IV, III-VI and mean percentage residual WBC concentration from baseline between pair wise subgroups- I-II, I-III, I-IV, I-V, I-VI, II-V, II-VI, was statistically significant.

The mean WBC concentration (PRP-2, Table-3) was lowest ($290.00 \pm 198.9 / \mu\text{L}$) with mean residual WBC concentration in PRP-2 from baseline $0.85 \pm 0.66\%$ at 9 min.

Table-3: Results of group-A samples after 2nd spin (Platelet and WBC concentration and recovery in PRP-2).

Subgroups	A-I (n=30)	A-II (n=30)	A-III (n=30)	A-IV (n=30)	A-V (n=30)	A-VI (n=30)
Mean platelet concentration prp-2 $\times 10^3 / \mu\text{L}$	737.90 ± 200.1	773.47 ± 196.6	771.17 ± 199.4	729.03 ± 225.4	671.03 ± 191.1	621.50 ± 189.2
Mean fold increase in platelet concentration in PRP-2 from PRP-1	1.72 ± 0.28	1.85 ± 0.27	1.98 ± 0.33	2.01 ± 0.38	1.98 ± 0.35	1.94 ± 0.31
Mean fold increase in platelet concentration in PRP-2 from baseline	3.84 ± 0.46	4.04 ± 0.43	4.02 ± 0.49	3.77 ± 0.57	3.50 ± 0.61	3.24 ± 0.62
Mean total platelet count in PRP-2 $\times 10^6$	737.90 ± 200.1	773.46 ± 196.6	771.16 ± 199.4	729.03 ± 225.4	671.03 ± 192.1	621.50 ± 189.2
Mean % platelet recovery in PRP-2 from PRP-1	91.39 ± 9.4	91.62 ± 8.2	92.71 ± 11.7	90.27 ± 13.5	86.07 ± 15.5	83.28 ± 13.4
Mean % platelet recovery in PRP-2 from baseline	76.80 ± 9.2	80.95 ± 8.7	80.57 ± 9.8	75.48 ± 11.5	70.12 ± 12.2	64.80 ± 12.5
Mean % residual platelet in PPP	1.76 ± 1.1	3.37 ± 4.6	4.83 ± 5.0	6.04 ± 3.3	7.68 ± 3.0	9.17 ± 5.0
Mean WBC concentration in PRP-2/ μL	2166.67 ± 1930.8	946.61 ± 907.7	467.00 ± 300.4	423.30 ± 578.7	303.30 ± 225.1	290.00 ± 198.9
Mean total WBC count in PRP-2 $\times 10^3$	2166.67 ± 1930.7	946.67 ± 906.7	467.00 ± 300.4	423.33 ± 578.7	303.33 ± 225.1	290.00 ± 198.9
Mean % WBC concentration in PRP-2 from baseline	5.93 ± 5.76	2.30 ± 1.92	1.36 ± 1.00	1.18 ± 1.59	0.87 ± 0.64	0.85 ± 0.66

3.2.2. For group-B:

After 2nd spin of group-B sample (PRP-2, Table-4), the difference among subgroups- B-I to B-VI of mean fold increase in platelet concentration in PRP-2 from PRP-1, mean fold increase in platelet concentration in PRP- 2 from baseline, mean percentage platelet recovery in PRP-2 from PRP-1, mean percentage platelet recovery in PRP-2 from baseline and mean residual platelet in PPP was statistically significant (All $P < 0.05$, ANOVA).

Pair wise *t*-test (PRP-2, Table-4) was performed between these subgroups, the difference in mean fold increase in platelet concentration in PRP-2 from PRP-1 between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, mean fold increase in platelet concentration in PRP-2 from baseline between pair wise subgroups- I-III, I-IV, I-V, I-IV, II-IV, II-V, II-VI, mean percentage platelet recovery in PRP-2 from PRP-1 between pair wise subgroups- I-II, I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, mean percentage platelet recovery in PRP-2 from baseline between pair wise subgroups - I-II, I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI and mean percentage residual platelet in PPP between pair wise subgroups- I-II, I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI, IV-VI, was statistically significant.

The mean percentage platelet recovery in PRP-2 from PRP-1 (PRP-2, Table-4) was highest $96.84 \pm 7.77\%$ with mean platelet concentration in PRP-2 $859.57 \pm 283.4 \times 10^3 / \mu\text{L}$ at 7 min. however mean platelet concentration was highest ($876 \pm 290.2 \times 10^3 / \mu\text{L}$) with mean fold increase in platelet concentration from baseline 4.08 ± 0.50 -fold and mean percentage platelet recovery from baseline $81.74 \pm 10.0\%$ at 17 min.

After 2nd spin of group-B sample (PRP-2, Table-4), the difference among subgroups- B-I to B-VI of mean WBC concentration, mean total WBC count in PRP-2, mean percentage residual WBC concentration in PRP-2 from baseline was statistically significant (All $P < 0.05$, Kruskal-Wallis H-test).

Pair wise Mann-Whitney U-test (PRP-2, Table-4) was performed between these subgroups, the difference in mean WBC concentration between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-IV, mean total WBC count between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-VI and mean percentage residual WBC concentration in PRP-2 from baseline between pair wise subgroups- I-III, I-IV, I-V, I-VI, II-IV, II-V, II-VI, III-V, III-VI, was statistically significant.

The mean WBC concentration (PRP-2, Table-4) was lowest ($323.33 \pm 233.9 / \mu\text{L}$) with mean percentage residual WBC concentration in PRP-2 from baseline $0.99 \pm 0.75\%$ at 6 min.

Table-4: Results of group-B samples after 2nd spin (Platelet and WBC concentration and recovery in PRP-2)

Subgroups	B-I (n=30)	B-II (n=30)	B-III (n=30)	B-IV (n=30)	B-V (n=30)	B-VI (n=30)
Mean platelet concentration prp-2 $\times 10^3 / \mu\text{L}$	740.97 ± 231.0	807.13 ± 271.7	837.23 ± 255.5	876.83 ± 290.2	869.03 ± 273.6	859.57 ± 283.4
Mean fold increase in platelet concentration in PRP-2 from PRP-1	1.64 ± 0.26	1.77 ± 0.35	1.90 ± 0.32	2.04 ± 0.35	2.08 ± 0.39	2.09 ± 0.41
Mean fold increase in platelet concentration in PRP-2 from baseline	3.47 ± 0.46	3.75 ± 0.50	3.92 ± 0.46	4.08 ± 0.50	4.08 ± 0.50	4.02 ± 0.50
Mean total platelet count in PRP-2 $\times 10^6$	740.96 ± 231.0	807.13 ± 271.7	837.23 ± 255.5	876.83 ± 290.2	869.03 ± 273.6	859.56 ± 283.4
Mean % platelet recovery in PRP-2 from PRP-1	84.50 ± 9.69	90.56 ± 7.15	93.05 ± 7.44	96.42 ± 6.83	96.54 ± 8.20	96.84 ± 7.77
Mean % platelet recovery in PRP-2 from baseline	69.5 1 ± 9.30	75.19 ± 10.00	78.58 ± 9.33	81.74 ± 10.00	81.73 ± 10.14	80.58 ± 10.03
Mean % residual platelet in PPP	7.91 ± 4.06	5.18 ± 3.91	4.16 ± 4.30	3.05 ± 3.20	2.10 ± 2.68	1.42 ± 2.14
Mean WBC concentration in PRP-2/ μL	1123.33 ± 1305.0	883.33 ± 1096.0	610.00 ± 613.2	476.66 ± 462.1	323.33 ± 233.9	356.66 ± 434.4
Mean total WBC count in PRP-2 $\times 10^3$	1123.33 ± 1305.0	883.33 ± 1095.7	610.00 ± 613.2	476.66 ± 462.1	323.33 ± 232.9	356.67 ± 434.4
Mean % WBC concentration in PRP-2 from baseline	3.08 ± 3.15	2.37 ± 2.38	1.67 ± 1.35	1.33 ± 1.00	0.99 ± 0.75	0.99 ± 0.97

4. Discussion:

Present study was designed to analyze stepwise recovery efficiency of platelets in PRP at different centrifugal force and time at 22°C (20°C-24°C) to find the optimal centrifugal force and time for PRP preparation.

During 1st centrifugal step in case of whole blood centrifugation, the centrifugal force and time drive the packing of erythrocytes at the bottom layer, WBC at middle layer and Platelets with volume of plasma at the upper layer. Our study result demonstrate it is not straight forward, it shows an exponential relationship with the distance travelled by cellular components during centrifugation. The RCF was constant for both the sample groups (700g for group-A and 200g for group-B) but centrifugal time was variable in increasing order (Figure-1). With increase of centrifugal time, supernatant plasma volume increases gradually (Table-1, 2), due to packaging of RBC towards bottom. WBC count in supernatant plasma decreases gradually (Table-1, 2), due to the movement and packaging of WBC towards middle layer. Platelet count with

percentage platelet yield in PRP-1 initially increases up to higher level and then decreases gradually (Table- 1, 2). The initial increase of platelet yield in plasma (PRP-1) shows initial movement of platelets from bottom layer to upper layer. This initial movement of platelet is due to their small size and low specific gravity relative to RBC. Platelets have a greater force of buoyancy (balancing force) in the bottom layer relative to RBC, which has a greater centrifugal force and moves toward the bottom layer. This movement of the platelets from the bottom to the radial axis i.e. supernatant plasma, results in increased platelet count and percentage platelet yield in PRP-1. With increase of centrifugal time, after equilibrium, plasma platelet has greater centrifugal force in the upper layer, results in the movement of the platelets from the supernatant plasma towards the middle layer, causing a gradual decrease of platelet count in PRP-1.

During the 2nd centrifugal step, applied centrifugal force (RCF) was constant for both the sample group (700g for group-A and 1500g for group-B) and time was variable (Figure-1). With the increase of centrifugal time, the mean percentage residual platelet in supernatant plasma (PPP) decreases gradually (Table-3, 4). It shows movement and packaging of the platelets towards the bottom, which results in the formation of the platelet pellet at the bottom of the test tube and recovery efficiency of platelets in PRP-2.

Our results shows, for 1st spin, centrifugation at 200g for 12 min at 22° C is an optimal condition, it produces PRP with lowest WBC contamination (0.90%) and a higher platelet recovery (84.63%). However highest platelet recovery (88.61) was observed at 700g for 3 min, it has higher residual WBC (3.10 %). For the 2nd spin, centrifugation at 1500g for 6 min is an optimal condition. It produces 96.54% platelet yield in PRP-2 from PRP-1 and leaves less residual platelet (2.10%) in PPP. Centrifugation at 700g for 15 min platelet recovery was 92.71% with higher residual platelet in PPP.

These findings explain centrifugal force and time plays an important role in PRP preparation, selection of centrifugal force and time for the 1st spin and 2nd spin separately affecting overall quantity of the platelets as well as the quality of PRP.

Araki *et al.* [66] reported same platelet recovery 70-80%, at 70g for 10 min and at 230-270g for 10 min, higher WBC recovery 10-35% at 70g for 10 min and lower 4.1%-5.8% at 230-270g for 10 min. They also reported, WBCs precipitated by higher centrifugal force ($\geq 840g$). In our study, the percentage platelet yield in PRP-1 was similar, at RCF-200 (table-4, 5) from 8 min to 13 min, but the percentage residual WBCs has significant difference and WBCs decrease with increase of centrifugal time, from 8 min (2.82%) to 12 min (0.90%). The WBCs decrease rapidly (Table-3) at RCF-700 (Higher RCF) from 5.94% (2 min) to 0.93% (7 min). Our result show WBC precipitate with an increase of the centrifugal force and centrifugal time also. WBC contamination can be minimized in PRP by adjusting, appropriate centrifugal force and time.

Rachita Dhurat and MS Sukesh [48] have reported, consistently platelet count more than $1 \times 10^6/mL$, after centrifugation at 16°C (low temperature), 1st spin at 900g (high RCF) for 5 min and 2nd spin at 1000g for 10 min, they homogenized platelets in the lower 1/3rd plasma. Amable *et al.* [67] have reported at 12°C (low temperature), 87% platelet recovery after 1st spin at 300g for 5 min and 97.4% platelet yield after 2nd spin at 700g for 17 min. They obtained 3.6-fold platelet yield in 300 μL PRP-2. But Macey *et al.* [49] reported cooling retard platelet activation and temperature is an essential factor for obtaining PRP with viable platelets. Transfusion medicine technical manual DGHS, [68] Standard for Blood Bank and Blood Transfusion Services NACO Govt. of India [69] and AABB manual recommends centrifugation at 20°C-24°C for obtaining PRP. [70] Considering these facts, we maintained temperature 22°C, during PRP Preparation and obtained 88.61% platelet yield after 1st spin at 700g for 3 min and 96.54% platelet yield in PRP-2 from PRP-1 after 2nd spin at 1500g for 6 min.

Jo *et al* [70] reported highest 92% platelet recovery (platelet concentration $310.7 \pm 78.5 \times 10^3/mm^3$) from 9 mL of whole blood, after 1st spin at 900g (higher RCF) for 5 min and after 2nd spin at 1500g for 15 min, highest 84% platelet recovery with 4.2-fold increased platelet concentration ($633.2 \pm 91.6 \times 10^3/mm^3$) from baseline. Dugrillon *et al.* [72] reported, the quality of PRP is more important than the quantity of the platelet. Growth factors TGF- $\beta 1$ increases with an increase of platelet concentration, when centrifugal force is less than 800g and decrease when forces are above 800g. In our study we observed a higher platelet yield at a higher RCF (highest 88.61% at RCF-700) than a lower RCF (highest 84.78% at RCF-200) after 1st spin.

Anitua E [27] proposed one spin method; they centrifuged 4.5 mL whole blood at 460g for 8 min and collected 0.5 mL of plasma located just above the Buffy coat as PRP with 2.67-fold increased platelet concentration. Amanda *et al* [73] reported, 70%-80% platelet recovery from 3.5 mL whole blood, after centrifugation 1st spin at 100g for 10 min and 2nd spin at 400g for 10 min. Landesberg *et al* [74] have reported, PRP with 3.2-fold increased platelet concentration from baseline value, after centrifugation of 5 mL whole blood, two times at 200g for 10 min per spin. Bausset *et al.* [75] reported that, centrifugation at 130g or 250g for 15 min was optimal for the two spins procedure. They obtained 2.0 mL of PRP with 3.47-fold increased platelet concentration, from the 8.5 mL of whole blood. Tamini *et al.* [76] compared two methods for obtaining PRP, in double centrifugation method (ACE-system) Platelet concentration was 336% after centrifugation of 8.5 mL whole blood, after 1st spin at 160g for 10 min and 2nd spin at 400g for 10 min. In single centrifugation (Nahita-System) method Platelet concentration was 227% after centrifugation of 3.5 mL whole blood at 280g for 7 min. Kececi *et al.* [77] have reported, after 1st spin at 250-270g for 10 min and the 2nd spin in increasing order- 300g, 500g, 750g, 1000g, 1500g and 2000g for 10 min, platelet concentration increased- 1.92-fold, 2.16-fold, 2.80-fold, 3.48-fold, 3.67-fold, and 3.76-fold. In most of these studies increase of platelet concentration is <4-fold or platelet recovery <80%. Our PRP preparation is highly purified and contains <1% WBC with increase of platelet concentration >4 -fold and >80% platelet recovery from baseline.

Many authors [37, 78-84] have specified revolutions per minute (rpm) instead of RCF in ×g for centrifugal accelerations. So comparing and reproducing their results are complicated, because rotors with different radius running at same rotational speed will exert different RCF on the sample. As per equation-

$$RCF (g) = (1.118 \times 10^{-5}) R S^2$$

(Where g is the RCF, R is the radius of the rotor in centimeters and S is the speed of the centrifuge in revolutions per minute)

This study may be helpful for personnel of blood transfusion medicine, researchers and clinicians of regenerative medicine to understand the complexity of the procedure and to prepare PRP with a standard quality and quantity of platelets, which can be used reliably in regenerative medicine and study evaluating regenerative ability of PRP in different regenerative processes.

5. Conclusion:

Different composition of platelets and WBCs in PRP can be prepared by adjusting centrifugal force and time. Centrifugation at 22°C for 1st spin at 200g for 12 min and 2nd spin at 1500g for 6 min was optimal condition for PRP preparation, for obtaining PRP with higher platelets and lower WBCs composition from 5 mL of whole blood.

6. Conflict of interest: Nil

7. Disclosures: None to disclose.

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