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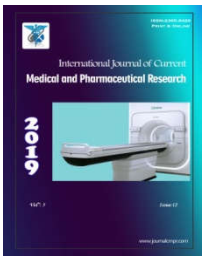

Research Article

EXPERIMENTAL RESEARCH ON A TECHNIQUE FOR QUANTIFICATION OF PLATELETS AND LEUKOCYTES IN SECOND-GENERATION PLATELET CONCENTRATES

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ABSTRACT

Neutrophil granulocytes and platelets are key players in wound healing and in combination with their secreted growth factors/cytokines can facilitate tissue regeneration and the formation of new blood vessels (angiogenesis). In a recent Kitamura Y. *et al*. Study, they showed a method for direct estimation of platelet counts in the PRF. These authors used a recombinant t-PA, through a digestion method. They showed that t-PA is powerful enough to be able to count the dispersed platelets aggregated in insoluble fibrin matrices enriched with platelets. Authors have elaborated the data obtained in the figures and tables provided, related to the work published by the Niigata University group, and have evaluated the percentage deviation between "counts with t-PA" and PLT and WBC values with "counts for subtraction "and with" counts for blood count "with the use of statistical techniques. By reducing the value of leukocytes in the PRF obtained by the subtraction method by 34.35%, the value reported with the t-PA method is obtained; reducing the value of the platelets in the PRF membrane obtained from the same method by 19.69%, the value reported with the t-PA method is always obtained; instead of reducing the value of leukocytes obtained from the blood count by 34.12%, the value reported with the t-PA method is obtained and reducing the value of the platelets obtained with the same method by 15.12%, the value reported with the method is obtained from digestion with t-PA. With this experiment the Authors made it possible to validate a simple and inexpensive system for calculating the precise number of platelets and leukocytes present in second-generation platelet concentrates, making it easy to evaluate individual PRF matrices in a timely manner in a clinical setting. It is also possible, with the same method, to evaluate the minimum/maximum level of platelets and leukocytes useful for having a clinical result in the use of Second Generation Platelet Concentrates.

INTRODUCTION

The multidisciplinary field of tissue engineering aims to repair, regenerate or restore damaged tissues in a predictable manner. Developed by Choukroun (2001), PRF (platelet-rich fibrin) has a simple, fast, cheap and free access technique that results in the formation of a fibrin clot rich in platelets and trapped leukocytes. L-PRF differs from other forms of Platelet Concentrates (HPCs) because its production protocol exploits the coagulation properties inherent in whole blood without the need for biochemical changes. Therefore, it denies the use of additives such as anticoagulants, thrombin, calcium chloride or synthetic preservatives. The original PRF protocol requires venous blood to be taken from the patient and deposited in 10 ml dry glass tubes. The PRF clot is intrinsically charged with platelets, leukocytes and growth factors. These growth factors begin to free themselves from the PRF matrix within 5-10 minutes of clot formation and continue to be released for 60-100 hours (Dohan Ehrenfest DM, *et al*. 2012). Schär *et al*. (2015) showed a constant release of growth factors and active stem cells for a period of 28 days. This indicates that the PRF clot is a reservoir of inactive and active cells capable of interacting with the cells and the molecules native to the site in which it is applied. A-PRF ™ (advanced PRF) has a relatively recent development based on a hypothesis which states that reducing the relative centrifugation force (force G), by reducing the centrifugation rate, would increase the number of

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leukocytes (in particular neutrophils and macrophages) within the PRF matrix (Ghanaati *et al*., 2014; Kobayashi *et al*., 2016). Choukroun (2014) states that A-PRF ™ was developed primarily in an attempt to include monocytes within the fibrin network because these cells play an essential role in bone growth, of vessels and in the production of two chemokines, that of the vascular endothelial growth factor (VEGF) and that of the bone morphogenic protein (BMP-2). Ghanaati *et al*. (2014) studied this concept and found that A-PRF ™ contains significantly more neutrophil granulocytes and their wider distribution in the fibrin clot than L-PRF. The group also found that, histologically, platelet distribution is more homogeneous throughout the A-PRF ™ clot than the L-PRF clot. It has also been shown that A-PRF ™ releases significantly greater total amounts of growth factors than L-PRF prepared at 2700 rpm (325G) for 12 minutes (Kobayashi *et al*., 2016). However, the A-PRF ™ processing protocol seems to have evolved within the literature. Previous studies show a centrifugation protocol of 1500 rpm (252G) for 14 minutes (Ghanaati *et al*., 2014; Kobayashi *et al*., 2016), while more recent studies have used a spin speed of 1300 rpm (189G) for 14 minutes for the production of A-PRF ™ and 1300 rpm (189G) for 8 minutes to produce A-PRF +. The current A-PRF ™ processing protocol involves the use of a new pre-programmed centrifuge (PRF DUO, PROCESS © for PRF, Nice, France), a standardized blood collection kit and a patented 10 ml vacuum tube of glass. The DUO centrifuge is able to produce the classic L-PRF, A-PRF ™ and lastly i-PRF ™ (injectable PRF ™).

Among most clinicians who study tissue regenerative therapy, it has generally been accepted that platelets are highly concentrated in buffy coat and are hardly present in other nearby fractions, particularly in the red blood cell (RBC) fraction, after fractionation through centrifugation.

This misunderstanding does not apply to the evaluation of the efficacy of platelet concentrations in liquid samples, which can be quantified with a simple blood count, but is especially extended to the evaluation of platelet counts in self-compressed platelet concentrates [(A-PRF, L-PRF and concentrated growth factors (CSFG)]. Because platelets have no nuclei, their count cannot be determined through DNA content. Therefore, to determine the platelet count in fibrin clots, a calculation is applied with the "subtraction method" (Dohan Ehrenfest, DM *et al*. 2010; Crisci A. *et al*. 2017) or "simulation" (Watanabe, T. *et al*. 2017) (Figure 1).

According to the subtraction method, the platelet count contained in fibrin clots is carried out by subtracting the number of platelets contained in the clot exudate (Surface post-compression), in the supernatant serum (PPP) and in the red blood cell fraction (i.e. the thrombus red) from those present in the initial whole blood sample. However, this method does not consider the possibility of the presence of platelets in the RBC fraction or the possible loss and damage of platelets during processing for cell counting.

To facilitate the carrying out of individual quality inspections, Kitamura Y. *et al*. (2018) developed a method to directly determine the platelet count in an insoluble PRF matrix using the tissue-plasminogen activator digestion procedure (t-PA Alteplase) (GRTPA®; Mitsubishi Tanabe Pharma Corp., Osaka, Japan). However, with this method, it is difficult to evaluate individual PRF matrices in a timely manner in a clinical setting. On the contrary, however, an effective standardization of preparation and practical application while not guaranteeing the quality of the individual PRF matrices is expected to effectively minimize variability and maximize efficacy in the same blood samples, consistent with other types of protocols of platelet concentration. The main advantages of the method proposed by Kitamura *et al*. they are: (1) high precision, (2) simple procedure, (3) non-technical skill and (4) no limitation to the types of fibrin matrix, while the main disadvantages are: (1) long completion times, (2) reagent cost (t-PA), (3) incubator requirements and (4) additional tubes required for growth factor analysis.

Therefore, in this study the authors, starting from the results obtained in the work of Kitamura, wanted to elaborate a simpler and inexpensive system to calculate the precise number of platelets and leukocytes present in the PRF, compared to that present in whole blood, starting from either the subtraction method that is from a simple blood count.

Leukocytes (WBC)		Dohan e al.(2010)	%	Crisci e al. (J. Unexplored Med. Data 2017)*	%	Watanabe e al. (MDPI 2017) A-PRF	%	Kitamura e al. (2018)	%
	WHOLE BLOODSHED	69 x 10^3 µl	100,00	51,5 x 10^3 µl	100,00	45,0 x 10^3 µl	100,00	36,22 x 10^3 µl	100,00
	RED CLOT	35 x 10^3 µl	50,7	0,085 x 10^3 µl	0,16	1,0 x 10^3 µl	2,22	n.r.	n.r.
	PRF	33,7 x 10^3 µl	48,8	51,1 x 10^3 µl	99,24	580,0 x 10^3 µl	?	26,73 x 10^3 µl	73,8
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,0002 x 10^3 µl	0,00	n.r.	n.r.	n.r.	n.r.
	SURFACE post compression	n.r.	n.r.	0,3 x 10^3 µl	0,58	1,5 x 10^3 µl	3,33	n.r.	n.r.
Red Blood Cells (RBC)									
	WHOLE BLOODSHED	5,19 x 10^6 µl	100,00	7,65x 10^6 µl	100,00				
	RED CLOT	5,8 x 10^6 µl	111,7	7,39 x 10^6 µl	96,6				
	PRF	0 x 10^6 µl	0,0	0,216 x 10^6 µl	0,0028				
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,002 x 10^6 µl	0,00				
	SURFACE post compression	n.r.	n.r.	0,013 x 10^6 µl	0,16				
Platelets (PLT)									
	WHOLE BLOODSHED	2,66 x 10^5 µl (266.000)	100,00	1,06 x 10^5 µl (106.780)	100,00	0,25 x 10^5 µl (25.000)	100,00	1,524 x 10^5 µl (152.400)	100,00
	RED CLOT	0,06 x 10^5 µl (6.000)	0,02	0,005 x 10^5 µl (500)	0,47	0,00 x 10^5 µl (000)	0,0	n.r.	n.r.
	PRF	2,6 x 10^5 µl (260.000)	97,7	1,05 x 10^5 µl (105.801)	99,00	3,2 x 10^5 µl (320.000)	?	1,27 x 10^5 µl (127.000)	83,3
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,0002 x 10^5 µl (2)	0,002	n.r.	n.r.	n.r.	n.r.
	SURFACE post compression	n.r.	n.r.	0,0048 x 10^5 µl (479)	0,45	0,00 x 10^5 µl (000)	0,0	n.r.	n.r.
Methods Used		Subtraction methods				Simulation method		Method for t-PA counting	

n.r.= not reported; *= values in the horse;

Figure 1 Comparison of platelet, leukocyte and erythrocyte counts with various methods.

MATERIAL AND METHODS

Starting from the study by Kitamura Y. *et al*. (2018) (Figure 2), the A.A. they wanted:

- Evaluates the percentage deviation between the t-PA count and the PLT and WBC values obtained with the "subtraction method", for which adding or subtracting the calculated percentage difference, to the value obtained with the subtraction method, we hypothesize to obtain the value derived with the t-PA method.

The procedure for counting with "subtraction method" is performed as described by Watanabe *et al*. (2017) according to the equation:

- PLT/WBC in A-PRF and L-PRF = PLT/WBC in whole blood - [(PLT/WBC in the red clot) - (PLT/WBC in serum over the PRF clot) - (PLT / WBC in the supernatant after compression of the coagulant PRF)]
- Evaluate the percentage deviation between the t-PA count and the PLT and WBC values obtained with a blood count (WB), for which adding or subtracting the calculated percentage difference, to the value obtained with a blood count, it is possible to have the value obtained with t-PA method.

The basic blood count is performed using test tubes with EDTA K3E 5.4 mg (VacuMed) and conducted with a Cell Dyn 3500 R (Abbott) Cell Counter.

The validity and precision of the measurements was evaluated through the coefficient of variation, considering the values valid if it is <2% on at least three measurements.

The zero hypothesis ($H_Ø$) formulated is that there is no statistically significant difference between the values measured with the t-PA method and the values with the "subtraction method" ± X% and between the values measured with the t-PA method and the measured values with the "blood count method" (WB) ± X%, both for PLT and WBC.
Formally $H_Ø$: t-PA = Met.Sottr. (PLT) (WBC) ± X%; t-PA = W.B. (PLT) (WBC) ± X%;

The relationship between the variables was evaluated by calculating the Pearson-Bravis Correlation Coefficient between t-PA and Met.Sottr. ± X% and between t-PA and W.B. ± X%, both for PLT and WBC, in the PRF.

The degree of agreement observed in the Correlation Coefficient was generally classified as: moderate if the coefficient r is between 0.65 and 0.8, good if the value is between 0.8 and 0.9 and optimal if it is> 0.9. A 95% confidence interval is used as a range of statistical significance.

To verify the zero hypothesis ($H_Ø$) the criterion Variance test (ANOVA) between t-PA and Met.Sottr is used. ± X% and between t-PA and W.B. ± X% for PLT and WBC in L-PRF and in other types of Platelet Concentrate (PC), then two other significance tests were used: the parametric Student's t-test and the Chi-square (χ^2).

The raw data detected with the t-PA counting method were provided by the Niigata University working group, Japan. From these we proceeded to the Statistical Analysis and to the Comparisons with the "method by subtraction" and with the "method by haemochrome" [4].

This calculation will justify statistically using Met.Sottr. ± X% and W.B. ± X% instead of t-PA in the evaluation of platelet and leukocyte counts in L-PRF and in various types of solid HPC, but with differences that is statistically significant between them (p≤0.000).

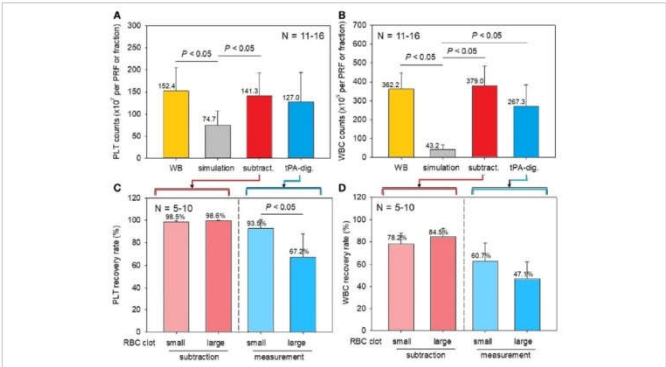

Figure 2 | A comparison of platelet and white blood cell (WBC) counting methods (simulation vs. subtraction vs. digestion). **(A,B)** Platelet and WBC counts in platelet-rich fibrin (PRF) clots and the corresponding fractions prepared from whole-blood samples (*N* = 11–6). **(C,D)** The relation between platelet and WBC counts in PRF clots and the sizes of red thrombi (small vs. large) (*N* = 5–10). (from Kitamura Y. e al.,2018 modified)

Statistical analysis

The results were calculated as Mean ± Standard Deviation (SD) and evaluated for significant differences at any time with the one-way variance analysis (ANOVA), the Student parametric test and the χ^2, using the Statistics software for Disciplines Biomedicals by Santon A. Glatz Ed. 2007 Version 6.0.

The differences were considered with p values lower than 0.05 (*) as significant and p values lower than 0.01 (**), 0.001 (***) and (p <0.0001 ****) as highly significant.

We also calculated the significance with the Wilcoxon and Friedman tests when there were results with Low Correlation to the Pearson-Bravis test.

RESULTS

The data provided by the Division of Oral Bioengineering, Institute of Medicine and Dentistry, Niigata University, Japan, are reported in Tables 1 and 2 and were statistically analyzed. The percentage deviation between the values obtained from the blood count (W.B.) and those of the same patient obtained with t-PA digestion was calculated. The value obtained for PLT is an average percentage deviation equal to -10.85 ± 26.01%. Since the results of the t-PA values for some were higher than the value of W.B., these values (in red) were excluded from a second calculation for the percentage deviation, obtaining an average deviation of -27.30 ± 15.82% (?).

Table 1 Method for counting with t-PA digestion. Prior to coagulation, PLT and WBC counts were detected directly (with blood count). After coagulation, PLT and WBC counts were determined with t-PA digestion. ?The second calculation derived from the hemochrome (W.B.) was performed by eliminating the values in red that are positive values with respect to W.B. (Data derived from the study by Kitamura Y *et al*, 2018).

The value obtained for WBC is an average percentage deviation of -22.74 ± 30.73%. Since, also in this case, for some values of t-PA higher than the value of W.B., these values (in red) have been excluded from the calculation of the percentage deviation obtaining an average deviation of -38.82 ± 16.03% (?).

We carried out the comparison between t-PA and W.B.-10.85% and W.B.-27.3% for PLT and between t-PA and W.B.-27.74% and W.B.-38.82% for WBC, carrying out Pearson-Bravis Correlation Coefficients.

Comparing the t-PA method and the blood count -% deviation for PLT, we have obtained that by subtracting 10.85% from the values of W.B. we have: t-Student test = 0.98 (Statistically Not Significant Difference) (NS); χ^2 = 1.000; ANOVA: 4.128 p = 0.97 (NS); Correlation Coefficient: r = 0.573; p = 0.065; which means Low Concordance between the values with Difference St.N.S.) *.

By subtracting 27.3% from W.B.: t-Student = 0.75 (NS); χ^2 = 0.005 (Statistically Significant Difference) (S); ANOVA: 115.4 p = 0.75 (NS);

Correlation Coefficient: r = 0.745; p = 0.054; which means Moderate Concordance between values (N.S.) *.

However, if we assume a significance of 0.001 ***, the difference values between t-PA and W.B.-27.3% are all statistically not significant. Therefore we can use the value of W.B. of PLT -27.3% to obtain the count values for t-PA with sufficient statistical appropriateness.

Comparing the t-PA method and the blood count -% deviation for WBC, we have obtained that by subtracting 22.74% from the WB values: t-Student = 0.96 (NS); χ^2 = 0.000 (S); ANOVA: 23.69 p = 0.96 (NS); Correlation Coefficient: r = 0.269; p = 0.423; which means Low Concordance between values (NS) *.

PLT counts	W.B. haem.	t-PA-digested	% deviance	W.B. -10,85±26.01%	W.B. □ -27,30±15.82%	WBC counts	W.B. haem.	t-PA-digested	% deviance	W.B. -22,74±30.73%	W.B. □ -38,82±16.03%
	157.48	108.60	31.04	140.39	114.48		355.60	136.50	61.61	274.73	217.45
	89.27	107.10	16.65	79.58			331.80	242.60	26.88	256.35	202.89
	100.80	91.10	9.62	89.86	73.28		400.00	314.00	21.50	309.04	244.60
	116.80	90.20	22.77	104.12	84.91		376.00	310.00	17.55	290.49	229.92
	261.63	132.80	49.24	233.24	190.20		583.20	312.00	46.50	450.58	356.62
x10^7/CGF	152.60	81.70	46.46	136.04	110.94	x10^5/CGF	287.00	184.00	35.89	221.73	175.50
	141.75	126.20	10.97	126.37	103.05		249.80	112.20	55.08	192.99	152.75
	190.76	217.80	12.42	170.06			342.00	431.80	20.80	264.22	
	197.34	251.10	21.41	175.92			343.20	451.50	23.99	265.15	
	96.60	76.30	21.01	86.11	70.23		378.00	205.00	45.77	292.04	231.14
	171.00	217.10	21.23	152.45			337.50	401.30	15.90	260.75	
mean	152.37	136.36	-10.85	135.83	106.73	mean	362.19	281.90	-22.74	279.82	
t-test Student		p>0.05		p>0.05	p>0.05	t-test Student		p>0.05		p>0.05	p>0.05
I.C.95%		da -35 a -67.01 p=0.52		da -49.3 a -48.3 p=0.98	da -43.9 a -32.4 p=0.75	I.C.95%		da -2.9 a -163.6 p=0.057		da -81.8 a -85.9 p=0.96	da -76.0 a -77.4 p=0.985
χ^2		85.54 p=0.000		81.25 p=1.000	18.44 p=0.005	χ^2		261.08 p=0.000		231.40 p=0.000	53.49 p=0.000
ANOVA				4.128 P=0.97	115.4 P=0.75	ANOVA				23.69 P=0.96	1.84 P=0.98

Instead, subtracting 38.82% from WB values we have: t-Student = 0.98 (NS); χ^2 = 0.000 (NS); ANOVA: 1.84 p = 0.98 (NS);

Correlation Coefficient: r = 0.695; p = 0.055; which means Moderate Concordance between values (NS) *.

If we assume a significance of 0.05 *, the difference values between t-PA and W.B.-38.82% are not Significant with a Moderate Concordance. Therefore we can use the value of W.B. of WBC -38.82% to obtain the count values for t-PA with sufficient statistical appropriateness.

The Wilcoxon pair examination between t-PA and W.B.-38.82% is W = -2.0 p> 0.054 (NS); Friedman test p = 1,000 (NS);

Other tests for t-PA, W.B. counts were subsequently performed on the same patients (Kitamura *et al*. 2008) and with the Subtraction Method, which are reported in Tables 2A and B.

Table 2. Count of Platelets and Leukocytes with "Method of counting with t-PA digestion" and "with Method of Subtraction". Before coagulation, the counts of PLT (A) and WBC (B) were detected directly (with blood count). After coagulation, PLT and WBC counts were determined by t-PA digestion and the Subtraction Method according to Watanabe *et al*. (2017) and Crisci e al (2017). The second calculation was performed by eliminating the values in red that are positive values compared to W.B. (Data derived from the study by Kitamura Y *et al*, 2018).

Comparing the t-PA method and the blood count -% deviation for PLT, we obtained that by subtracting 20.43 ± 21.44% from WB values we find: t-Student = 0.83 (NS); χ^2 = 0.000 (S); ANOVA: 155.9 p = 0.83 (NS);

Correlation Coefficient: r = 0.92; p = 0.000; which means Excellent Concordance (S) **** (Table 2A).

A

PLT counts	W.B. haem.	t-PA-digested	Method Sottraz	% deviance tra WB e Sottr.	W.B. -20,43±21.44%	W.B. □ -25,09±20.42%	% deviance tra t-PA e Sottr.	Met.Sottr. -19,69±21.88%	Met.Sottr. □ -24,31±21.02%
	155.00	126,20	154.50	18.58	123.33	116.11	18.32	124.07	116.90
	132.50	108.60	130.90	18.04	105.40	99.25	17.04	105.10	99.07
	117.80	107.10	112.40	9.08	93.73	88.24	4.72	90.26	85.07
	228.40	217.10	227.90	4.95	173.30	171.09	4.74	183.03	172.49
	245.60	217.80	245.60	11.32	195.42	183.97	11.32	196.72	185.89
x10^7/CGF	248.50	251.10	247.60	1.04	197.73		1.39	198.32	
	126.70	132.80	125.50	4.59	100.81		5.50	100.52	
	104.60	91.10	103.00	12.91	83.23	78.35	11.55	82.50	77.96
	100.70	81.70	100.00	18.87	80.12	75.44	18.30	80.10	75.69
	108.20	34.10	108.20	68.48	86.09	81.05	68.48	86.66	81.89
	136.60	76.30	136.60	44.14	108.69	102.33	44.14	109.41	103.39
	144.90	80.40	144.90	44.51	115.29	108.54	44.51	116.06	109.67
mean	154.13	127.03	153.09	-20.43	121.93	110.44	-19.69	122.73	110.80
t-test Student		p>0.05	p>0.05	p>0.05	p>0.05	p>0.05		p>0.05	p>0.05
I.C.95%		da -24.7 a -78.9 p=0.29	da -45.6 a -47.7 p=0.96	da -78.0 a -25.9 p=0.31	da -42.4 a -42.6 p=0.826	da -43.4 a -50.6 p=0.874		da -43.7 a -52.4 p=0.85	da -44.1 a -50.5 p=0.88
χ^2		54.14 p=0.000	0.118 p=1.000	55.29 p=0.000	51.345 p=0.000	38.731 p=0.000		50.972 p=0.000	40.024 p=0.000
		ANOVA			155.9 P=0.83	64.91 P=0.87		110.7 P=0.85	52.4 P=0.89

B

WBC counts	W.B. haem.	t-PA-digested	Method Sottraz.	% deviance tra W.B. e Sottr.	W.B. -45,9±16.67%	% deviance tra t-PA e Sottr.	Met.Sottr. -34,35±16.67%
x10^5/CGF	350.0	112.20	251.20	67.94	189.35	55.33	164.91
	426.3	136.50	272.30	67.98	230.60	49.87	178.76
	380.0	242.60	271.00	36.16	205.58	10.48	177.91
	667.3	401.30	575.50	39.86	361.00	30.27	377.81
	616.0	431.80	559.70	29.90	333.25	22.85	367.44
	592.8	451.50	489.60	23.84	320.70	7.78	321.42
	563.2	312.00	458.80	44.60	304.69	32.00	301.20
	410.8	314.00	389.60	23.56	222.24	19.40	255.77
	420.8	184.00	319.30	56.27	227.65	42.37	209.62
	385.4	126.50	334.30	67.18	208.50	62.16	219.46
	469.2	205.00	404.40	56.31	253.84	49.31	265.48
	462.0	290.40	417.20	37.14	157.11	30.39	273.89
mean	478.65	267.32	395.24	-45.90	251.21	-34.35	259.47
t-test Student		p>0.05	p>0.05		p>0.05	p>0.05	p>0.05
I.C.95%		da -306.3 a -116.4 p=0.00	da -174.5 a -7.659 p=0.071		da -64.8 a -97.0 p=0.68	da 30.73 a -225.1 p=0.012	da -75.6 a -91.3 p=0.84
χ^2		165.82 p=0.000	28.343 p=0.003		163.44 p=0.000	110.824 p=0.000	93.09 p=0.000
			ANOVA		3344.0 P=0.59		1413.0 P=0.73

By comparing, instead, the t-PA method and the Subtraction Method -% of deviation for PLT, we have obtained that reducing by 19.69 ± 21.88% the values obtained with Subtraction Met. We have: t-Student = 0.85 (NS); χ^2 = 0.000 (S); ANOVA: 110.7 p = 0.85 (NS); Correlation Coefficient: r = 0.92; p = 0.000; which means an Excellent Concordance (S) **** (Table 2A). For the calculation of PLT the differences between t-PA and W.B.-20.43% and t-PA and Met.Sottr.-19.69% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.0001 **** the difference values are not significant with an Excellent Concordance.

Since values of t-PA were found for some higher than the value of W.B., these values (in red) were excluded from the calculation of the percentage deviation obtaining an average deviation of -25.09 ± 20.42%.

Comparing the t-PA method and the blood count -% deviation for PLT, we have obtained that by subtracting the 25.09 ± 20.42% to the WB values: t-Student = 0.874 (NS); χ^2 = 0.000 (S); ANOVA: 64.91 p = 0.87 (NS); Correlation Coefficient: r = 0.94; p = 0.000; which means Excellent Concordance (S) **** (Table 2A).

Comparing, instead, the t-PA method and the Subtraction Method -% of deviation for PLT, we have obtained that by reducing by 24.31 ± 21.02% to the values of the Subtraction Method: t-Student = 0.88 (NS); χ^2 = 0.000 (S); ANOVA: 52.4 p = 0.89 (NS); Correlation Coefficient: r = 0.91; p = 0.000; which means Excellent Concordance (S) **** (Table 2A). For the calculation of PLT the differences between t-PA and W.B.-25.09% and t-PA and Met.Sottr.-24.31% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.0001 **** the difference values are not significant with an Excellent Concordance.

Furthermore, comparing the t-PA method and the blood count -% deviation for WBC, we have obtained that by subtracting 45.9 ± 16.67% from the WB values: t-Student = 0.68 (NS); χ^2 = 0.000 (S); ANOVA: 3344.0 p = 0.59 (NS); Correlation Coefficient: r = 0.73; p = 0.007; which means Moderate Concordance (S) *** (Table 2B).

Comparing the t-PA method and the Subtraction Method -% deviation for WBC, we have obtained that by reducing by 34.35 ± 16.67% to the values of the Subtraction Method: t-Student = 0.84 (NS); χ^2 = 0.000 (S); ANOVA: 1413.0 p = 0.73 (NS); Correlation Coefficient: r = 0.88; p = 0.000; which means Good Concordance (S) **** (Tables 2B, 3). For the calculation of WBC the differences between t-PA and W.B.-45.9% and t-PA and Met.Sottr.-34.3% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.001 *** the difference values between t-PA and WB-45.9% for WBC are not Significant with a Moderate Concordance. If, instead, we assume a significance of 0.0001 **** the values of difference between t-PA and subtraction-34.35% for WBC are Not Significant with a Good Concordance.

For this comparison the Wilcoxon pair examination between t-PA and W.B.-45.9% was W = 16.0 p> 0.052 (NS); Friedman test p = 0.564 (NS);

Subsequently we added the values obtained in Tables 1, 2A and 2B exclusively for the comparison values between t-PA and W.B. and the results obtained would seem to be the most valid Statistically (t-PA and WB-15.12 ± 24.87% for PLT and between t-PA and WB-34.12 ± 28.2% for WBC (Table 3).

Comparing the t-PA method and the blood count -% deviation for PLT (Table 3), we obtained that by subtracting 15.12% from the WB values: t-Student = 0.93 (NS); χ^2 = 0.000; (S); ANOVA: 10.45 p = 0.953 (NS) *; Correlation Coefficient: r = 0.766; p = 0.000, with Good Concordance between the values (S). The t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 demonstrates statistically significant differences together with the correlation coefficient. If we assume a significance of 0.0001 **** the difference values between t-PA and W.B.-15.12% are all not Significant. This means that we can use the WB value of PLT - 15.12% to obtain the count values for t-PA with sufficient statistical accuracy.

Comparing the t-PA method and the blood count -% deviation for WBC (Table 3), we obtained that by subtracting 34.12%

from the WB values: t-Student = 0.88 (NS); χ^2 = 0.000; (S); ANOVA: 217.6 p = 0.88 (NS) *; Correlation Coefficient: r = 0.47; p = 0.022; which means Poor Concordance between values (S). The t-test and ANOVA and the Correlation Coefficient demonstrate statistically non-significant differences, while χ^2 demonstrates statistically significant differences. If we assume a significance of 0.0001 **** the difference values between t-PA and W.B.-34.12% are all not Significant. Since the comparison between the t-PA and WB-34.12% methods for WBC has a Low Correlation Coefficient (r = 0.47; p = 0.022) with a Statistically Significant Difference, we proceeded to calculate the significance with Wilcoxon tests and Friedman between the values of t-PA and WB-34.12%, WB-45.9% and WB-38.82%.

Based on the comparison with the Wilcoxon and Friedman tests, the calculation of W.B.-34.12% for WBC is considered valid.

From this we can deduce that we can use the WB value of WBC- 34.12% to obtain the count values for t-PA with sufficient statistical accuracy.

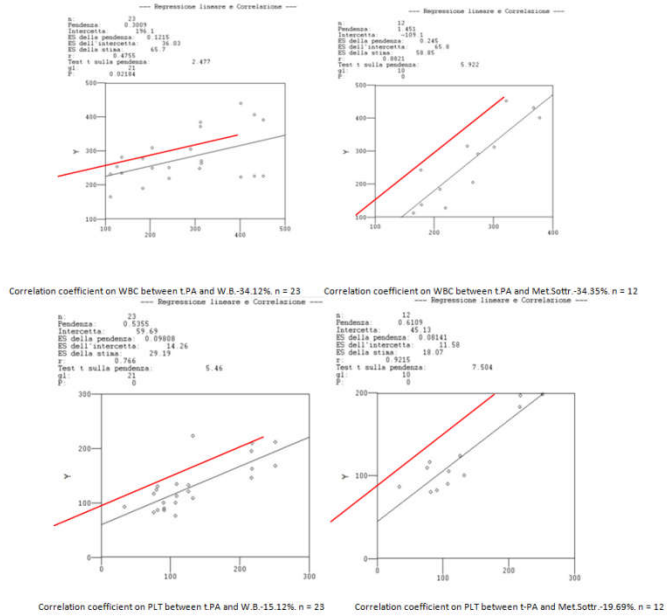

Figure 3 PLT and WBC Correlation Coefficient between t-PA, W.B. and Subtraction Method.

WBC counts x10^5/CGF					PLT counts x10^7/CGF			
t-PA-digested	W.B. haem	W.B-t-PA	W.B. - 34,12±28.2%		t-PA-digested	W.B. haem	W.B-t-PA	W.B. - 15,12±24.87%
136.50	355.60	61.61	234.27		108.60	157.48	31.04	133.67
242.60	331.80	26.88	218.59		107.10	89.27	-19.97	75.77
314.00	400.00	21.50	263.52		91.10	100.80	9.62	85.56
310.00	376.00	17.55	247.71		90.20	116.80	22.77	99.14
312.00	583.20	46.50	384.21		132.80	261.63	49.24	222.07
184.00	287.00	35.89	189.08		81.70	152.60	46.46	129.53
112.20	249.80	55.08	164.57		126.20	141.75	10.97	120.32
431.80	342.00	-26.26	225.31		217.80	190.76	-14.17	161.92
451.50	343.20	-31.56	226.10		251.10	197.34	-27.24	167.50
205.00	378.00	45.77	249.03		76.30	96.60	21.01	81.99
401.30	337.50	-18.90	222.35		217.10	171.00	-26.96	145.14
112.20	350.00	67.94	230.58		126.20	155.00	18.58	131.56
136.50	426.30	67.98	280.85		108.60	132.50	18.04	112.47
242.60	380.00	36.16	250.34		107.10	117.80	9.08	99.99
401.30	667.30	39.86	439.62		217.10	228.40	4.95	193.87
431.80	616.00	29.90	405.82		217.80	245.60	11.32	208.47
451.50	592.80	23.84	390.54		251.10	248.50	-1.05	210.93
312.00	563.20	44.60	371.04		132.80	126.70	-4.81	107.54
314.00	410.80	23.56	270.64		91.10	104.60	12.91	88.78
184.00	420.80	56.27	277.22		81.70	100.70	18.87	85.47
126.50	385.40	67.18	253.90		34.10	108.20	68.48	91.84
205.00	469.20	56.31	309.11		76.30	136.60	44.14	115.95
290.40	462.00	37.14	304.37		80.40	144.90	44.51	122.99
274.29	422.95	34.12	278.64	Mean	131.49	153.28	15.12	130.11
115.31	110.75	28.20	72.96	±D.S	63.45	52.26	24.87	44.36

DISCUSSION

Platelet concentrates are safe, reliable and cost-effective means of accelerating tissue healing and improving tissue repair efficiency after injury.

The platelets, fibrin and leukocytes contained in them act naturally in synergy to promote wound healing and tissue regeneration. The concept of platelet concentrates for surgical use is to multiply this coagulation / regeneration effect on a surgical site or wound.

Despite the clinical use of PRF in the last 15 years, no research to date has managed to quantify the number of platelets and leukocytes present in this and other platelet concentrates in a direct, simple and effective way, in order to relate it to their regenerative potential. The presence of leukocytes also has a great impact on wound healing biology, not only because of their additional release of growth factors and their implications for antibacterial immune defense but also because it is the key regulators that control wound healing through the local regulation of growth factors. Future basic research should focus specifically on the contribution of these cells to specific cell knock-down/knock-in systems to determine the functional roles of each cell type in the wound healing process when PRF is used. Thus, in theory, the concept of developing new modified PRF protocols to further increase the number of white blood cells would lead, in principle, to increase wound repair. Nevertheless, a better understanding of the individual roles of the various cells found in PRF could prove to be an important discovery for the development of these technologies, which leads to modern changes in their protocols and to further increase their regenerative potential.

The number of platelets is a quantity, but it has been accepted as one of the main indexes to guarantee the quality of the platelets presents (Marx, 2001). However, up until current studies, there is lack of a method for the accurate determination of platelet counts in gel types.

Platelet concentrates, including PRF and A-PRF, have been used, in fact, for regenerative procedures in various fields of medicine, including dentistry, reconstructive plastic surgery and dermatology, to provide supraphysiological concentrations of autologous growth factors directly to guest fabrics. These growth factors have been shown to be chemotactic for various cell types, including monocytes, fibroblasts, endothelial cells, stem cells and fibroblasts, creating tissue microenvironments and directly influencing the proliferation and differentiation of progenitor cells.

The beneficial effect of PRF membranes in complex wound healing can be explained by the high concentration of platelets and leukocytes along with long-term release of specific growth factors.

It is clear that platelet-rich fibrin clots form a bioactive reservoir. Therefore, a high hematocrit or a low number of platelets may be a limiting factor and this is why further research is needed to establish the optimal number of platelets for their clinical use. The cytokines produced by platelets and leukocytes are therefore an important part in the role of this biomaterial, but both the fibrin matrix and the elements contained in it are responsible for the real therapeutic enhancement of the PRF. Until now it was almost impossible to count and regulate the number of platelets in PRF

preparations before their clinical use, so as to put it in relation to the result for each patient. Therefore, the most clinically effective way to control the quality of the results was to use the PRF region closest to the RBC clot.

material, therefore, there is an increased risk of transmission of infectious agents.

3. The number of cells (platelets, leukocytes and stem cells) contained can be very variable.

A value of P less than 0.05 was considered a Statistically Significant Difference; *p>0.05 Statistically Not Significant Difference;
n.r.= not reported;

Figure 4 Results obtained between t-PA, Subtraction and Simulation counting methods

Activated platelets release a full range of chemokines and promote the uptake, adhesion and proliferation of adult stem cells, including CD34-positive progenitor cells, MSCs, SMC progenitors, and endothelial progenitors. The leukocytes present in L-PRF are not only inflammatory cells, as they also present anti-nociceptive effects through different chemokines, anti-inflammatory cytokines (IL-4, IL-10, IL-13) and opioid peptides (β-endorphins, dimorphine -A etc.) and therefore can promote a clinically relevant inhibition also of pathological pain. The released amounts of VEGF and TGF-β1 are produced massively by leukocytes. A significant correlation between platelet number and release of TGF-β1 ($p = 0.005$) and PDGF-BB ($p = 0.04$) was detected.

Wend *et al*. (2017) showed that a decrease in PRF centrifugation speed leads to a greater number of leukocytes, lymphocytes, neutrophils, monocytes and platelets in it, compared to the higher PRF generated at RCF (Applied centrifugal force). Thus, the amount of growth factor and cytokine release from PRF matrices increases with decreasing RCF forces. The pro-angiogenic effect of PRF is also significantly greater when a low-RCF PRF is used compared to the PRF with high RCF and can be related to the presence of a large number of trapped extracellular neutrophils and neutrophils (NET) that are released.

The limitations, however, found in the clinical use are therefore:

1. due to the fact that PRF is an autologous product, the availability of this biomaterial in greater quantity is difficult. Therefore, its use in surgical procedures must be well controlled.
2. the PRF possesses circulating immune cells and antigenic molecules that prevent its use as an allogeneic

At this point of our knowledge, among the important parameters to be taken into account we therefore have: the concentration of platelets, the concentration of leukocytes and the proportion between the various types of leukocytes. Regarding the concentration of platelets, leukocytes and their formula, their influence on the clinical effect of second-generation platelet concentrates, has yet to be studied carefully, as their more or less abundant presence can explain the contradictory results that were observed in the published works.

CONCLUSIONS

In this study it will be possible to deduce that, subtracting the value of the Platelet count by the subtraction method from the Emocrome value by 34.35%, and by 19.69% the value of the Leucocyte count by subtraction Method and by 34.12% value of the blood count, the value obtained with the method of digestion from t-PA will be obtained with a much simpler system. From this study it will therefore be possible to validate a simple and inexpensive system for calculating the precise number of platelets and leukocytes present in platelet concentrates. The present study has shown that it is indeed possible to quantify the number of cells in biomaterials, a complex cellular system due to the presence of platelets, leukocytes, stem cells etc., using a clinical method that can be applied quickly (max 15 minutes) and safely.

With this, it will be possible to evaluate the minimum/maximum level of platelets and leukocytes useful for having a clinical result in the use of Second Generation Platelet Concentrates.

Other experimental and clinical studies must be conducted to provide a better understanding of the cross-talk between the

number of platelets, leukocytes and the mechanisms that control tissue repair, characterized by processes such as the recruitment of cells with regenerative potential and the regulation of apoptosis/cell survival.

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