Chapter 6
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Estimation of Cellular Content in Second-Generation Solid Fibrin Concentrates (PRFs)

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ABSTRACT

We performed a study to evaluate the amount of Platelets and Leukocytes in a second-generation solid platelet concentrates in terms of Sensitivity, Specificity, Positive Predictive Value (VP+), Negative Predictive Value (VP-), False Negative Proportion, and False Positive Proportion of our Statistical Method. Blood was collected in anticoagulant-free PET tubes with silica for clot production and PRF membranes. The membranes and clots produced were examined. In a previous work, the authors, starting from the results obtained in Kitamura's work, wanted to develop an easier and inexpensive system to calculate the exact amount of platelets and leukocytes contained in PRF, compared to the one present in whole blood, starting from a simple "haemochromocytometric examination". In this study, the authors have evaluated the Sensitivity, Specificity, Positive Predictive Value (VP+), Negative Predictive Value (VP-), False Negative Proportion, and False Positive Proportion of the "Statistical Method". Using the Statistical Method, we have this result by reducing by 34.12% (±28.2) the value of leukocytes obtained from the CBC examination. We obtained the value of leukocytes contained in the PRF membrane by t-PA digestion and by reducing by 15.12% (±24.87) the value of platelets obtained by the same method, we have reached the value contained in solid platelet concentrates by t-PA digestion. The proposed method proved a Se=0.75; Sp=0.86; VP+=0.75; VP-=0.86 for platelet counts, Se=0.47; Sp=0.66; VP+=0.80; VP-=0.30 for leukocyte counts. Conclusion: Our study tried to standardize the PRF preparation procedure by validating a statistical system to calculate the exact amount of platelets and leukocytes in second-generation solid platelet concentrates, making it easy to evaluate individual PRF arrays on time in the clinical setting. The Statistical method compared to the digestive method with t-PA for leukocyte and platelet counts proved to be equally valid for platelets, but not in the same way for leukocytes.

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1. INTRODUCTION

Recently, "second-generation" Platelet-rich fibrin (PRF) was suggested as the novel bioregenerative therapeutic strategy to promote healing of soft tissue [1,2]. PRF or leukocyte- and platelet-rich fibrin (L-PRF) is obtained from patients' blood and typically centrifuged at a relative spin force (RCF)-max/G-force of 800 for 12 minutes without any additives [3,4].

PRF not only works like a three-dimensional fibrin scaffolding but also includes multiple autologous cells, such as platelets, macrophages, and neutrophils, and stem cells [5].

The ability of platelets to release substances from within a clot (PC-Platelet Concentrate) makes the latter a natural autologous source of growth factors and cytokines that can be used in the clinic to accelerate the physiological healing of a tissue injury [1,2].

Because leukocytes, embedded neutrophils and macrophages are in the top cell types present at injury sites, their functions include removing phagocytic fragments, bacteria and necrotic tissue, thereby preventing infection. Neutrophils, along with platelets and in association and their excreted growth promoters/cytokines, can facilitate tissue regeneration, generation of newly formed blood vessels (angiogenesis), with prevention of infection (bacteriostasis) [6].

Various types of second-generation concentrates have been produced by centrifuging blood drawn in different ways:

-L-PRF**:** 30" of acceleration, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm and 36" of deceleration and stopping [1];

-*Advanced-PRF*: 1300rpm, 8 minutes (189 g-forces) [7];

-*Injectable-PRF*: 700 rpm (60 g-forces) per 3 minutes [8];

-*Horizontal-A-PRF*: 1300 rpm (189 g-forces) per 8 minutes [4];

Recently, the authors have studied an L-PRF, which appears to contain hematopoietic stem cells (HSCs) [5]. The presence of these HSC cells was detected mainly by immunohistochemical analysis to detect specific cellular markers CD34.

A recent study by Kitamura Y. et al. [9] shows a procedure for directly platelet count estimation in PRF. These authors utilized a professional available method recombinant t-PA, Alteplase (GRTPA®; Mitsubishi Tanabe Pharma Corp., Osaka, Japan) via a digestion method. Using this fairly elaborate procedure, they demonstrated that t-PA is sufficiently potent to succeed in counting dispersed platelets aggregated in platelet-enriched insoluble fibrin matrices (Table 1).

Table 1. Comparison of Method for Count with t-PA digestion and CBC for WBC and PLT [9-10,16,23]

WBC count x10^5/CGF					PLT count x 10^7/CGF			
t-PA-digested	**W.B. blood**	**W.B-t-PA**	**W.B.-34,12±28.2%**		**t-PA-digested**	**W.B. blood**	**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_

Recently, AA. (2019, 2022) have elaborated, in collaboration with Kawase's Japanese group, a statistically valid mathematical method to predict the presence of cells in PRF starting from the performance of a blood thrombocytometric examination (Table 1) the validation of a simple and costless method to quantify the exact number of platelets & leukocytes contained in platelet concentrates [10].

2. MATERIALS AND METHODS

Starting from the study by Kitamura Y. et al. [9], the AAs aimed to: evaluate Se (Sensitivity), Sp (Specificity), VP+ (Positive Predictive Value), VP- (Negative Predictive Value), False-Negative Proportion, and False-Positive Proportion of the two methods (digestion with t-PA and Statistic) compared (Table 2).

Table 2. Applied tests on statistical evaluation of cell count

Proportion False Negatives = It is the fraction of test negatives
Proportion False Positives = It is the fraction of test positives
Sensitivity(If**)**= Indicates the odds of obtaining a true positive result
Specificity (Sp**)**= Indicates the probability of obtaining a false-positive result
Positive Predictive Value (*VP*+)= Indicates the odds that a test + is actually positive (True Positive)
Negative Predictive Value (*VP*-)= Indicates the probability that a test - is actually negative (True Negative)

Blood was collected in tubes without anticoagulant or PET gel separator with silica for clot production and PRF membranes (Vacuaptaca clot activator tubes cod.30023).

When PRF preparation protocols are described in the literature, there are no indications of important parameters generally (e.g., if anticoagulant is used and its volume, volume of blood collected and final PDP reached, working temperature, the time elapsed between collection/processing/analysis of samples), which leads to questionable and not comparable results.

In our protocol, blood was rapidly collected with a 19 G needle in 9 cc vacutainer tubes using a vacuum system (average collection rate of 22", less than 25" per tube) and immediately (after two minutes of rest with the test tube stable) centrifuged according to the above description at a temperature over 21°C.

Adopted processing temperature range 21.3/22.5°C.

Currently, to maintain the temperature of the test tubes and test tube holders of the centrifuge in a suitable way, the AA. used a constant temperature incubator apparatus (Fig. 1).

Cold processing (<20°C) of clots and membranes leads to pauperisation of the biological and physical characteristics of the product and the ideal minimum temperature for storage for some hours is +4°C (no more than 2 hours).

Fig. 1. Incubator used by the authors

In addition, this was not designed as a clinical study and the control group is represented by the results obtained with the count with t-PA digestion.

A blood sample was also collected to execute a blood enumeration utilizing K3E 5.4 mg tubes with EDTA (VacuMed). Leukocyte and platelet concentrations of L-PRF were analyzed with a CBC in standard bays and performed with a Cell Dyn 3500 R cell counter (Abbott Laboratories; Abbott Park, IL, USA).

AA., in a recent study [10], elaborating the data gained by Kawase's study group [9] obtained that, by reducing by 15.12% the importance of the platelet counting from the Hemochrome and by 34.12% the importance of the leukocyte count, they derived the value reached by the digestion method with t-PA [9] with a much simpler system. Therefore, they experimented with an easy and economical system to indirectly calculate the number of platelets and leukocytes contained in self-compressed platelet concentrates (not liquid). The study has shown that it is possible to estimate the number of cells in solid biomaterials, a complex cellular system for the presence of platelets, leukocytes, stem cells, etc., using a clinical method applicable in a rapid (max 15 minutes) and safe way (Statistical Method). As confirmed in the current research study, the authors wanted to review the results by evaluating the parameters of Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value of the Statistical Method, comparing them with the same parameters achieved with the Method of Digestion with t-PA.

2.1 Statistical Measurements and Analysis

Data elements were investigated using version 6.0 of the Santon-Glantz 2007 Statistical Package for Biomedical Disciplines. Sensitivity and Specificity for platelet and leukocyte counts were calculated on the data obtained with t-PA by the Japanese Group (Aureo test) and on the data gained with the "Statistical Method" by AA (WB-15.12% for platelets and WB- 34.12% for WBC), considering as cut-off the average value of count with t-PA digestion (131.49 *vs.* 274.29). In

addition, Positive and Negative Predictive Values (VP+, VP-) and the Proportion of False Negatives and Positives were calculated on the same results (Table 2).

The validity and accuracy of the measurements were assessed by the coefficient of variation, considering the values valid if it is <2% over at least three measurements.

The zero hypothesis ($H_{\varnothing}$) formulated is without statistically significant difference between values measured by "t-PA method" and values measured by the "blood count method" (W.B.) ± X%, for both PLT and WBC.

Formally $H_{\varnothing}$ *: t-PA =W.B.(PLT)(WBC) ± X%;*

3. RESULTS AND DISCUSSION

The membrane and clot preparation procedure was well tolerated in all examined subjects.

No significant differences were detected in the baseline hematology comparison of the examined subjects, which had a mean RBC concentration of 5.6×10^6/mL (±1.1 I.C.95 %) (p=0.34) (range:4-6×10^6/mL), of WBC of 5.1×10^3/mL (±0.37 I.C.95%) (p=0.24) (range:4.3- 10×103/mL) and a Mean Platelet Count of 176.8×10^3/mL (±15.3 I.C.95%) (p=0.15) (range:150-350×10^3/mL).

The values were confronted with the Statistical Indirect Count Method.

In a suggestive phase of the experimentation, we compared the platelet and leukocyte count method with the t-PA digestion proposed by Kawase [9] with the Statistical method presented in 2019 [10], whose values are shown in Table 1. In this study, we were able to show that, by reducing by 15.12% (±24.8) the amount of the platelet counting to the amount of the CBC, and by 34.12% (±28.2) the number of the leukocyte counting to the amount of the CBC, we will obtain the value achieved with the method of digestion with t-PA.

We constructed the values obtained with the two methods, then the ROC (Relative Operating Characteristic) curve for PTL and WBC for values gained with *t-PA dig* and with W.B.-15.12% for PLT and -34.12% for WBC.

The cut-off considered is represented by the average t-PA for PLT (131.49) and WBC (274.29) (Fig. 2 A-B).

On these values, we calculated, in the simplest hypothesis in which it is assumed that the "gold test" provides results perfectly corresponding to the truth, the Sensitivity (Se), the Specificity (Sp), the Positive Predictive Value (*VP+*), the Negative Predictive Value (*VP-*), the Proportion of False Negatives and the Proportion of False Positives (Tables 2, 3 and 4). We have therefore assumed as a "gold test" the one resulting from the *t-PA dig* count.

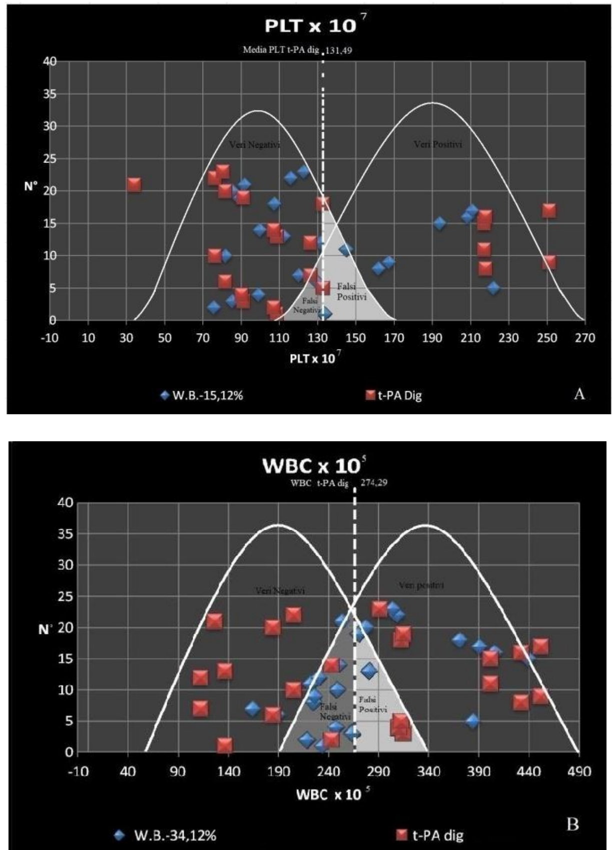

Fig. 2. ROC curve for (A) PTL and (B) WBC for values obtained with *t-PA dig* and with W.B.-15.12% for PLT and -34.12% for WBC. The cut-off is represented by the mean t-PA for PLT (131.49) and for WBC (274.29)

The results obtained showed for PLT a Sensitivity of 0.75 (Gold Standard=0.80), Specificity of 0.86 (Gold Standard=0.86), for WBC a Sensitivity of 0.47 (Gold Standard=0.80), Specificity of 0.66 (Gold Standard=0.75).

Table 3. Contingency table for PLT. The "Gold Standard" was referred to as the *t-PA dig* test

PLT Aureus Test (*t-PA dig*)

	M.	N.M.	Tot.	Off Scale		
Pos	4	2	6	0	*Se*=0.80	*VP+*=0.66
Neg	1	13	14	0	*Sp*=0.86	*VP-*=0.92
Tot	5	15	23	3	Prop.False Neg =0.2	Prop.False Pos =0.13

Test PLT (WB-15.12%)

	M.	N.M.	Tot.	Off Scale		
Pos	6	2	8	0	Se=0.75	VP+=0.75
Neg	2	13	15	0	Sp=0.86	VP-=0.86
Tot	8	15	23	0	Prop.False Neg=0.25	Prop.False Pos=0.13

Table 4. WBC contingency table. The "Gold Standard" was referred to as be the *t-PA dig* test

Golden test WBC (t-PA dig)

	M.	N.M.	Tot.	Off Scale		
Pos	8	3	11	0	Se=0.80	VP+=0.75
Neg	2	9	11	0	Sp=0.75	VP-=0.81
Tot	10	12	23	1	Prop.False Neg=0.2	Prop.False Pos=0.25

Test WBC (WB-34.12%)

	M.	N.M.	Tot.	Off Scale		
Pos	8	2	10	0	Se=0.47	VP+=0.80
Neg	9	4	13	0	Sp=0.66	VP-=0.30
Tot	17	6	23	0	Prop.False Neg=0.53	Prop.False Pos=0.33

Also a *VP*+ of 0.75 (Gold Standard=0.66), *VP*- of 0.86 (Gold Standard=0.92) for PLT. A *VP*+ of 0.80 (Gold Standard=0.75), *VP*- of 0.30 (Gold Standard=0.81) for WBC. The Proportion of False Negatives is 0.25 for PLT (Gold Standard=0.2) and 0.53 for WBC (Gold Standard=0.2). The Proportion of False Positives is 0.13 for PLT (Gold Standard=0.13) and 0.33 for WBC (Gold Standard=0.25).
The PRF group, adapts to the needs of a diversity of surgical procedures.

Like clots and membranes, PRF has a shape and volume that is simple to associate with many others surgical procedures, including the following filling and interposition of healing biomaterials or as protective membranes for injury treatment. Finally, it is easy to prepare in medium quantities and inexpensive, making it suitable for everyday clinical practice. It was used to benefit humans by AA., particularly in treating diabetic skin ulcers, including chronic osteomyelitis [11-13]. The study performed by McLellan et al. [14] demonstrated that equine PRF, similar to human PRF, is an instantaneous and sustained resource of tissue growth factors. Our trial sought to confirm the validation of the "Statistical Method" by standardizing the procedure for the preparation of L-PRF, which, while remaining an easy technique to perform at low cost and requiring no specialized equipment, has some consolidation in the generation of an L-PRF membrane.

L-PRF and its derivatives represent remarkable progress in the evolving platelet concentrates. It is essentially a fibrin matrix membrane containing platelets and

leukocytes entrapped in combination with stem cells. These rigid films exhibit exceptional handling properties and can be sutured securely into an anatomically desired position in open surgery procedures.

Although, the physical and biological characteristics are virtually unknown and yet have to be fully investigated.

Based on these results, it is evident that L-PRF is a revolutionary new bio-material with unique features: -foreseeable preparation from self blood; -simple protocol; -defined architecture; -impressive mechanical properties; -and plenty of growth promoter factors derived from activated platelets.

Our tests performed on equine blood are undoubtedly capable of improving the comprehension of mechanisms of healing and advancing the field of personalized medicine.

Their influence should be studied with particular attention concerning leukocyte count and concentration, as their presence or absence could explain the conflicting results noted in multiple trials [15-27].

The results obtained in this study on the sustainability of the "Statistical" counting of platelets and leukocytes showed for PLT a Sensitivity of 0.75 (Gold Standard=0.80), Specificity of 0.86 (Gold Standard=0.86), for WBC a Sensitivity of 0.47 (Gold Standard=0.80), Specificity of 0.66 (Gold Standard=0.75).

4. CONCLUSIONS

Concluding this research, we can state that the preparation of PDPs is extremely technically challenging due to the delicate nature of platelets that are frequently initiated during the procedure. Consequently, special considerations are needed to maximize the therapeutic benefits of these blood products. Today, their procurement is supported by the poorly controlled bulk release. As a result, prolonged treatments require multiple treatments. This results in large fluctuations in Platelet, Leukocyte, and Stem Cell concentrations, compromising clinical predictability. Hence the need to experiment with simple methods for counting, even indirectly, contained cells, as biomaterials can act as controlled-release devices, allowing prolonged or even on-demand administration of these cocktails with the addition of growth factors.

This experimentation indicates it is useful to use the WB value of PLT -15.12% to obtain the values of count for t-PA with sufficient statistical accuracy with a Sensitivity of 0.75 *vs.* 0.80 and a Specificity of 0.86 *vs.* 0.80, therefore very similar to the "Gold Test". Using instead, the value of WB of WBC- 34.12% has a Sensitivity and Specificity much lower than the "Gold Standard" (Sensitivity of 0.47 *vs.* 0.80, Specificity of 0.66 *vs* 0.75).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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