J Bras Patol Med Lab, v. 53, n. 6, p. 382-387, December 2017

10.5935/1676-2444.20170061

Pseudothrombocytopenia: incidence and strategy for resolution in clinical laboratory

Pseudotrombocitopenia: incidência e estratégia para resolução em laboratório de análises clínicas

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ABSTRACT

Introduction: Pseudothrombocytopenia is an important source of pre-analytical problems in clinical laboratory; therefore it must be correctly evaluated. Low spurious platelet counts can lead to misdiagnosis. **Objective:** This study aims to study the resolution of pseudothrombocytopenia in the laboratory routine of clinical laboratory by standardizing the use of vortexer. **Methods:** This is a cross-sectional, analytical, quantitative study based on experimental approach, involving 45 pseudothrombocytopenia samples divided into three groups and submitted to vortexing for 1, 2 and 3 minutes. **Results:** During the study, 28,435 blood counts were performed, and 56 (0.196%) of these samples confirmed platelet aggregates. The 2 and 3 minute times normalized the platelet count (greater than 150,000/µl) in two thirds of the samples tested. Platelets, hemolysis index, platelet distribution width (PDW) and mean platelet volume (MPV) presented statistically significant mean difference (MD) ($p < 0.05$) after vortexing at all times. **Conclusion:** The 2 and 3-minute times were the most effective in the resolution of pseudothrombocytopenia, however, the 2 minutes time should be preferred because it produced fewer effects on the erythrocyte membrane.

Key words: thrombocytopenia; platelets counting; clinical laboratory techniques; laboratory automation.

INTRODUCTION

Platelets are figurative elements of the circulating blood of granular and discoid aspects, arising from the fragmentation of megakaryocytes. An individual under normal physiological conditions which has a mean blood count of 223,000 platelets/µl, and values between 150,000/µl and 400,000/µl are considered normal[1, 2]. Low platelet count or thrombocytopenia can be defined as platelet counts less than 150,000/µl, applicable for healthy individuals in all age groups, as well as those having different etiopathogenesis, such as insufficient production (aplasia or chemotherapy), excessive consumption (disseminated intravascular coagulation) and destruction or increased splenic sequestration (viruses with antiplatelet antibodies)[2, 3].

Several preanalytical or analytical conditions and factors may lead to falsely reported thrombocytosis or thrombocytopenia, so-called pseudothrombocytopenia[2]. The Brazilian Society of Clinical Pathology and Laboratory Medicine [Sociedade Brasileira de Patologia Clínica e Medicina Laboratorial (SBPC/ML)] presents several situations that promote overestimation of platelet counts, such as hemolysis, cryoproteins and the presence of fungi and/or bacteria; and underestimation, due to the formation of platelet aggregates, platelet satellites and inappropriate collections[4].

Platelet counts may still be affected by *in vitro* or *in vivo* hemolysis, which generates erythrocytes fragments that may have platelet-like dimensions and are sometimes mistaken for platelets, which leads to an overestimation of their count when performed by the electrical impedance method. Some hemocytometers equipped with fluorescence or optical channel for platelet counting are not subject to interference from fragmented red blood cells (RBC) due to the use of optical scanning method. Among the markers of hemolysis, the Determination of Lactate Dehyfrogenase (HDL) activity, the evaluation of the presence of schistocytes in a peripheral blood smear and increased indirect bilirubin, can be used, and the latter should not be used as a marker of *in vitro* hemolysis[2, 5, 6].

First submission on 10/07/17; last submission on 05/12/17; accepted for publication on 05/12/17; published on 20/12/17

In vitro platelet aggregation is a great example of pseudothrombocytopenia. Although it is difficult to estimate, studies that considered all possible forms of pseudothrombocytopenia show incidence between 0.1 % and 2% in outpatients and 1.9% in intensive care unit (ICU) patients. In a study conducted in Brazil with 56,337 samples, 117 presented pseudothrombocytopenia, corresponding to 0.2%[3, 7-9].

In addition to venipuncture accidents that cause platelet aggregates, there is also ethylenediamine-tetraacetic acid (EDTA)-induced pseudothrombocytopenia. However, it has been related to the production of antibodies that bind to antigens present in the platelet membrane, which are modified or exposed by the action of this anticoagulant. It is believed that these antibodies are directed against GPIIb/IIIa complex in up to 83% of cases, and immunoglobulin class M (IgM) or G (IgG). Although it is called EDTA-induced pseudothrombocytopenia, this phenomenon can occur in the presence of other anticoagulants, such as citrate and oxalate[3, 4, 10].

Pseudothrombocytopenia is an important source of pre-analytical problems in the clinical laboratory, so it is always necessary to raise suspicions about its possibility and to use correction methods. Repeated releases of platelet results below the reference value have led to misdiagnosed suspicion of disease, and may subject the patient to investigations, procedures and even to unnecessary treatments[2, 9].

Platelet indices such as mean platelet volume (MPV) may help in the definition of true thrombocytopenia. MPV is calculated by dividing the plateletcrit (PCT) by the total number of platelets[11] and represents their mean size, while the platelet distribution width (PDW) indicates a mean variation in their size. With the dissolution of the aggregates, the MPV and the PDW tend to decrease, since this process will directly decrease the mean platelet size and smaller size variation, as in cases of Von Willembrand's disease[10]; and will be increased in true thrombocytopenia, in which there is consumption or sequestering, and in several other inflammatory conditions, such as ankylosing spondylitis and rheumatoid arthritis[12]. It is important to note that in platelet aggregates due to pseudothrombocytopenia, the MPV will also be falsely elevated.

Once identified in the laboratory, a low platelet count requires the use of some methods to establish the correct diagnosis. Guidelines recommend heating for ten minutes at 37ºC, performing a new blood collection using anticoagulant different from the one that generated the event and using a vortex mixer to attempt dissolution of platelet aggregates[4]. Platelet aggregates are flagged by automated blood counters through specific alerts. SBPC/ML considers as the gold standard for confirmation of pseudothrombocytopenia the observation of the stained blood smear, evidencing the presence of isolated and/or clustered platelet aggregates in several regions of the slide[4, 13].

The literature is scarce on the subject of pseudothrombocytopenia and diverges in terms of the standardization of time and speed variables necessary and sufficient for the use of vortex for dissolution of the aggregates, without this implying greater erythrocyte fragmentation and falsely elevated results. SBPC/ML recommends a time of three minutes (without speed setting), whereas in a study conducted by the hematology group of Fleury laboratory, a two minutes time of agitation was enough to solve 85.5% of the cases. None of the studies estimated the speed used in the device or considered the consequence of this agitation on the erythrocyte membrane and the generation of hemolysis and schistocytes[4, 9].

OBJECTIVES

This study aims to study the resolution of pseudothrombocytopenia in the routine of the clinical laboratory by standardizing the use of vortex in a controlled experiment.

METHOD

This is a cross-sectional, analytical and quantitative study, presenting an experimental approach based in analysis of blood samples with decreased platelet counts with microscopic evidence of platelet aggregates. The study was carried out in the LABO® Análises Clínicas, a private clinical laboratory, based in the city of Vitória da Conquista, Bahia, Brazil. The sampling of this study consisted of whole blood samples collected in EDTA from patients with complete blood count (CBC) and/or platelet count ordering, who had pseudothrombocytopenia confirmed by blood smear and who had sufficient volume for analysis.

After pseudothrombocytopenia confirmation in the slide and demonstration of platelet aggregates, platelet counts were performed using the Fonio method, and the samples were submitted to the following analyzes, according to **Figure**.

Samples selected were divided into three groups of 15 samples. The tubes were vortexed at 3000 revolutions per minute (rpm) for 1, 2 and 3 minutes times. A control experiment was performed with 15 platelet samples without platelet aggregates, which were also submitted to the same rotation of 3000 rpm for 3 minutes, in

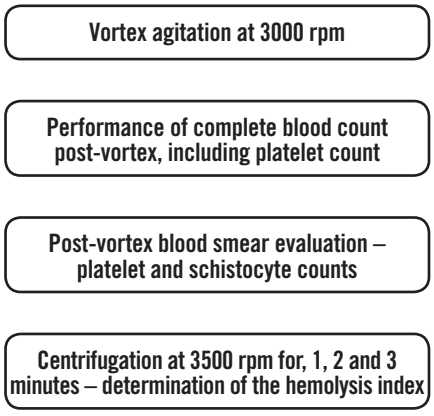

FIGURE – *Flowchart showing methodological aspects of the experiment*
rpm: revolutions per minute.

order to control the increase in platelet count due to erythrocyte fragmentation.

The vortex device was calibrated using a tachometer, having the speed established and set at 3000 rpm. Platelet counts and other hematological parameters were measured on Cell Dyn Rubi, Abbott Diagnostics®. The hemolysis index (HI) was determined by the VITROS® 4600 Ortho Clinical Diagnostics integrated system, through a solid-state spectrophotometer that uses dissonant diffraction to determine the HI, using the derivative of the absorption spectra.

The variables used for analysis of vortex efficiency and their effects on the erythrocyte membrane were: platelet count, percentage of schistocytes, MPV, PDW, hemoglobin, RBC, plateletcrit (PCT) and HI. All parameters were determined before and after vortex use. HI pre-vortex was determined in patient serum samples from the same venous puncture. The variables used in the control group were platelets, MPV, PDW, RBC and hemoglobin.

The percentage of schistocytes was determined by the smear count stained by standardized technique in the SlideInk® device brand Hemogram, which uses the Wright dye. The schistocyte count was performed by optical microscopy, and the results presented according to the formula: PS = NS*100/1000, where PS is the percentage of schistocytes and NS the number of schistocytes in 1000 red blood cells.

For statistical analysis, the Microsoft Office Excel® 2011 and the Statistical Package in Social Science® [(SPSS) version 21] programs were used. The variables were evaluated through mean difference and 2-tailed paired Student's *t*-test. A value of $p < 0.05$ was adopted for statistical significance and considered 95% of confidence interval (CI).

The study was approved by the Research Ethics Committee of the Health Multidisciplinary Institute (Instituto Multidisciplinar de Saúde) of the Universidade Federal da Bahia (UFBA) under number 1.839.064, dated November 28, 2016. All ethical precepts in the research were respected in the present study.

RESULTS

During the study 28.435 CBC were performed, of which 56 (0.196%) were confirmed with the presence of platelet aggregates. From these, 45 were selected for analysis and compose the *n* of this study. The remaining samples were excluded because they had insufficient volume or had no other serum or plasma samples. The 2 and 3 minute times were the most effective in normalize the platelet counts (greater than 150.000/µl) in 10 samples (66.6%). However, the two minutes time was able to completely solve the aggregates (absence of aggregates in stained slide) in eight (53.3%) samples, while the three minutes time solved six samples (40%).

The pre-vortex platelet count had the lowest mean in the two minutes time (**Table 1**); the highest post-vortex mean for the platelet variable was also observed in the two minutes time. The HI and the schistocyte count had a higher post-vortex mean in the three minutes time. The HI showed a statistically significant difference ($p < 0.05$) in all times studied. These observations demonstrate the efficiency of vortex mechanical agitation in increasing platelet counting and their correction to normal levels. The increase in platelets was accompanied by a decrease in MPV and PDW which also showed significant differences. This difference corroborates the identification of the resolution of the platelet aggregates.

Among the studied variables, platelet, HI, PDW and MPV presented a statistically significant mean difference ($p < 0.05$) after vortexing at all times tested (**Table 2**). The difference in the mean of the schistocyte count was statistically significant only in the three minutes time. However, there is a tendency to increase the count of schistocytes when increasing time. The other variables did not show statistical significance for p value < 0.05.

Mean hemoglobin also appears to show increase at the 2 and 3 minutes time, although not statistically significant. The greatest increase in platelet counts was observed in the two minutes time, in which the biggest difference in the MPV, PDW and the leukocyte count was also denoted. The percentage of schistocytes increases proportionally with the increase in vortex

TABLE 1 – Analysis of central tendency and dispersion of pre- and post-vortexing studied variables

	Before vortexing						After vortexing					
	1 minute		2 minutes		3 minutes		1 minute		2 minutes		3 minutes	
Variables	AM	SD	AM	SD	AM	SD	AM	SD	AM	SD	AM	SD
Platelets (/µl)	119.9	28.42	78.41	34.12	110.9	22.92	167.2	54.85	184.72	85.57	161.2	34.93
Schistocytes (%)	0.01	0	0	0	0	0	0.03	0.12	0.38	0.52	0.7	0.3
Hemoglobin	13.51	1.85	13.51	1.79	12.83	1.2	13.45	1.96	13.73	1.8	13.07	1.19
Erythrocytes (/µl)	4.76	0.69	4.81	0.77	4.47	0.38	4.77	0.73	4.85	0.84	4.48	0.34
MPV (fl)	9.89	2.19	9.98	1.54	9.64	1.78	8.03	2.02	8.47	1.27	8.27	1.87
PCT (%)	0.12	0.04	0.11	0.04	0.128	0.01	0.12	0.03	0.18	0.14	0.15	0.02
PDW (%)	22.03	1.24	22.99	1.03	21.5	1.2	20.52	1.26	21.34	1.77	20.11	1.55
Platelets* (/µl)	248.4	79.7	230.1	39.08	187.8	28.65	268.4	83.97	262	67.86	194.93	23.61
Hemolysis HI	26	19.96	19.73	9.13	15.8	1.82	66.66	35.66	115.53	41.52	149.8	29.75

MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet distribution width; HI: hemolysis index; AM: arithmetic mean; SD: standard deviation.
**: platelet counting by the Fonio method.*

TABLE 2 – Statistical evaluation of vortex efficiency in the dissolution of platelet aggregates and alteration of constants and hematimetric indices

	1 minute – 3000 rpm			2 minutes – 3000 rpm			3 minutes – 3000 rpm		
Variables	MD	T	p^*	MD	T	p^*	MD	T	p^*
Platelets (/µl)	-18.933	-3.578	0.003	-64.139	-5.407	0	-27. 652	-4.76	0
Schistocytes (%)	0.084	1.000	0.334	-0.097	-2.86	0.013	-0.533	-8.966	0
Hemoglobin	0.359	0.407	0.69	-0.048	-2.628	0.02	-0.055	-2.788	0.015
Erythrocytes (/µl)	0.111	0.181	0.859	0.025	-1.415	0.179	0.026	-0.88	0.394
Leukocytes (/µl)	178.11	-0.008	0.994	1390.12	0.324	0.75	853.239	1.317	0.209
MPV (fl)	-0.984	-4.530	0	2.163	4.993	0	1.755	7.478	0
PCT (%)	0.013	-0.750	0.466	0.011	-1.85	0.086	0.069	-1.617	0.128
PDW (10 GSD)	-0.844	-4.902	0	2.196	6.429	0	2.008	4.788	0
Platelets** (/µl)	9.92	-1.434	0.174	1.98	-2.019	0.063	4.892	-1.272	0.224
Hemolysis HI	-64.052	3.73	0.002	-71.259	-8.372	0	-117.42	-17.337	0

*MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet distribution width; HI: hemolysis index; rpm: revolutions per minute; MD: mean difference; T: Student-*t *test.*
: statistical significance* $p < 0.05$; *: platelet counting by the Fonio method.*

time. The three minutes time presented a significant increase in the HI and in the statistically significant schistocyte count ($p < 0.05$). This result indicates the occurrence of mechanical hemolysis[6].

The HI presented a statistically significant mean difference in all the times tested, behaving linearly, which shows a tendency to increase with increasing vortex time. The fact that it is significant already in one minute and the appearance of schistocytes in a slide in only three minutes is related to the sensitivity of the method used, derived spectrophotometry, available in the VITROS® 4600 Ortho Clinical Diagnostics device. It is a technique with high sensitivity in the detection of hemolysis and high selectivity through signal increase and noise reduction[14].

In the control experiment there was no statistically significant mean difference for the parameters considered in this study, including the platelet count, which presented a mean difference of 9,167 platelets/µl. It can be inferred, from this data, that, although the production of schistocytes occurs, the erythrocyte fragments did not interfere statistically in the mean platelet count.

DISCUSSION

The prevalence of pseudothrombocytopenia in this study is in line with a previous study by Mourad *et al*. (2011)[9], which presented an incidence of 0.2% and also demonstrated that the mechanical agitation with vortex for two minutes solve 85% of cases of low platelets, without, however, controlling the rotation speed or the interferents, such as erythrocyte fragmentation.

In the present study, the two minutes time at 3000 rpm solved a lower proportion of cases compared to the Mourad *et al*. (2011)[9] study. However, limiting the lack of control between collection time and sample analysis and failure to evaluate other of thrombocytopenia causes, such as that induced by anticoagulants with IgG or IgM antibodies that may have strong binding[10], may have underestimated the effectiveness of the method.

It should be pointed out that the thrombocytopenia not resolved by vortexing agitation were not evaluated or investigated as to their origin or pathophysiology due to the objective of the study to constitute only the vortex methodology analysis. This fact may have underestimated the resolution percentage of pseudothrombocytopenia cases, since additional resolution techniques can be used to resolve pseudothrombocytopenia, such as heating the sample for 10 minutes at 37ºC and ordering a new collection in anticoagulant different from the one that generated the disturbance.

It is still listed as a limitation the schistocytes counting, which, since it is a manual technique, is subject to the observer bias. Nevertheless, the high HI in three minutes time corroborates greater damage to the erythrocyte by mechanical agitation. It is also worth mentioning the existence of haemocytometers equipped with optical laser channel that do not suffer interference from the presence of schistocytes, so they would not suffer interference from the speed or the vortex agitation time for correction of platelet counting, even though it has a higher cost.

This study was carried out in relation to the possible spurious increase in platelet count due to the counting of erythrocytes fragments, which was confirmed by the lack of significant differences between the variables studied in the control experiment.

CONCLUSION

The development of the present study allowed the determination of a standardized and controlled procedure for the use of vortex in the dissolution of platelet aggregates, recommending the use of the equipment at 3000 rpm for two minutes. In addition, it has been shown that vortex use has an impact on the membrane, and the time and rpm variables should be controlled for satisfactory results. The recommended time and the rpm in this study were the ones that caused less impact on the erythrocyte Erytrocyte membrane and the dissolution of platelet aggregates in most cases. It is also worth noting the need for periodic calibration of vortex device, as well as other laboratory instruments, according to legal requirements.

The results obtained in this work present a fast, controlled and effective method for the dissolution of platelet aggregates, which allows laboratory workers greater support for results release, avoiding unnecessary collection and preventing the patient from undergoing unnecessary medical ivestigations and procedures.

ACKNOWLEDGEMENTS

To the LABO® Análises Clínicas for allowing this study to be carried out in its premises.

RESUMO

Introdução: *A pseudotrombocitopenia constitui importante fonte de problemas pré-analíticos no laboratório clínico, por isso deve ser corretamente avaliada. Baixas contagens de plaquetas espúrias podem levar a suspeitas diagnósticas equivocadas.* ***Objetivo:*** *Este estudo se propôs a estudar a resolução da pseudotrombocitopenia na rotina de laboratório de análises clínicas pela padronização do uso de vortex.* ***Métodos:*** *Trata-se de um estudo transversal analítico e quantitativo, apresentando-se dentro de uma abordagem de cunho experimental, o qual envolveu 45 amostras pseudotrombocitopênicas divididas em três grupos e submetidas a agitação com vortex nos tempos de 1, 2 e 3 minutos.* ***Resultados:*** *Durante a realização do estudo, foram realizados 28.435 hemogramas, sendo 56 (0,196%) amostras confirmadas com a presença de agregados plaquetários. Os tempos de 2 e 3 minutos normalizaram a contagem de plaquetas (maior que 150.000/µl), em dois terços das amostras testadas. Plaquetas, índice de hemólise, amplitude de distribuição das plaquetas (PDW) e volume plaquetário médio (VPM) apresentaram diferença de média estatisticamente significativa (*$p < 0,05$*) após o tratamento com vortex em todos os tempos testados.* ***Conclusão:*** *Os tempos de 2 e 3 minutos foram os mais eficazes na resolução da pseudotrombociopenia, contudo, o tempo de 2 minutos deve ser utilizado por ter produzido menores efeitos sobre a membrana eritrocitária.*

Unitermos: *trombocitopenia; contagem de plaquetas; técnicas de laboratório clínico; automação laboratorial.*

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