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Original article

Evaluation of survival and functionality in frozen platelets

Jhon Alexander Avila Rueda [a,b,*], *Jose Acosta* [a], *Oscar Rabinovich* [a], *José Ceresetto* [a], *Glenda Ernst* [a], *Cristina Duboscq* [a]

[a] *Hospital Británico de Buenos Aires; Buenos Aires, Argentina*
[b] *Hospital Privado Santa Clara De Asís, Salta, Argentina*

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ABSTRACT

Objective: Prolong platelet survival and functionality up to 28 days.
Methods: A sample of apheresis platelets was evaluated, distributed in 3 groups according to the cryopreservative solution used: DMSO5%+2%albumin; DMSO5%+NaCl0,9% and DMSO5%+Dextrose2%. They were then frozen at -80 °C and thawed at 7, 14 and 28 days. The in vitro survival and viability were assessed by the post-thaw platelet count and the CD41, CD61 and CD42a staining percentages by flow cytometry. The functionality was determined with the percentage of post-stimulation aggregation with 1Nm-thrombin using the Chromo-Log490 aggregometer. The control group (CG) consisted of fresh platelets under constant agitation at 22 °C.
Results: A total of 72 platelet aliquots was analyzed. The CG presented a platelet-count of $1934 \pm 0.5 \times 10^9$/L and a 100% viability. The percentages of CD41, CD61 and CD42a labeling were 99, 98.5 and 96.5%, respectively. The percentage of aggregation was 99%. On day 7 of the post-freezing, the platelet count for groups 1, 2 and 3 was 1,844 ± 102, 1,856 ± 76 and 1,752 ± 226, with the viability of 98, 96 and 95%, respectively. On day 14, the counts were 1,722 ± 238, 1,649 ± 215 and 1,578 ± 223 with the viability of 96, 95 and 94% and, on day 28, they were 1,602 ± 374, 1,438.6 ± 429 and 1,406.6 ± 436, with the viability of 96, 94 and 93%, respectively. Group1 presented a higher expression of membrane antigens. Aggregation percentages were 90, 98 and 89% at day 7, 88%, 98 and 87% at day 14 and 84%, 95 and 82% at day of the 28 post-freezing, respectively, with group2 presenting the best results.
Conclusion: The results support cryopreservation as a reasonable method to prolong platelet survival up to 28 days, maintaining its functionality and viability greater than 50%.

Introduction

The transfusion of blood components currently represents a resource of vital importance, given its usefulness in different clinical stances, either as support treatment or, in the case of critical patient care, as the first line of management to

* Corresponding author at: Specialist in Internal Medicine and Hematology, British Hospital of Buenos Aires, Hematology and Hemotherapy service, Perdriel 74, Autonomous, Buenos Aires, Argentina.
E-mail address: jaavilar@gmail.com (J.A.A. Rueda).
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guarantee hemodynamic stability.[1,2] Platelets are part of this group of blood components, whose use is extended to the management of a variety of pathologies related to the different medical-surgical specialties, making it essential to guarantee the constant provision of this component in medium- and high-complexity health institutions.[3-6]

As a result of the health emergency caused by COVID19, a significant decrease has been reported in the number of people who attend hemotherapy centers and transfusion medicine services as blood or blood component donors, which translates into a progressively lower availability of blood components, including platelet concentrates.[7-9]

That is why strategies have been sought that allow the optimization of this resource, one of them being platelet cryopreservation, using additive solutions based on dimethyl sulfoxide (DMSO) at 5%, whose investigations have reported promising results regarding the period of conservation and viability of processed platelets.[10-14]

An example of this is the work related to cryopreservation and usefulness of the use of frozen platelets in transfusion medicine, mainly by the United States, United Kingdom, Spain, the Netherlands, Australia, the Czech Republic and Turkey.[2]

The freezing method used in these tests corresponds totally or partially to that described by Valeri and collaborators in the 1950s. This strategy consisted of obtaining with the apheresis technique a concentrate with at least 3.3×10^9 platelets in a 200-mL solution, mixed with 75 mL of 25% DMSO solution, resulting in a final DMSO concentration of 5 or 6% for each platelet concentrate studied. Subsequently, the solution obtained should be centrifuged at 3600 rpm for 20 min and the supernatant eliminated (removing excess DMSO content), obtaining a final product that contains the platelets that were cryopreserved in a freezer at −80 °C.[1,15]

As there is no relevant research on the use of this strategy in Latin America,[3] this study seeks to use the cryopreservation method to prolong the survival time of platelets for up to 28 days, compared to those preserved under the conventional method, maintaining a viability equal to or greater than 50%, using different cryopreservative solution alternatives.

Materials and methods

A blind prospective study was performed. Aliquots of platelets with group and factor A+, A-, B+, B-, AB+, AB-, O+ and O- were included, obtained from voluntary donors over 18 years of age who expressed their decision to participate in this protocol by signing an informed consent for this purpose.

Platelets were collected by apheresis from all of them using the Trima ACCEL cell separator, version 7.0 marks TERUMO BCT, guaranteeing the attainment of a number of platelets greater than or equal to 3.3 × 1011 in a volume of 200 ml (equivalent to 1650×10^9 per liter).

From each bag, 3 ml were stored in sterile cryobags after the cryopreservative solution had been added. These aliquots were randomly distributed in each of the 4 groups described below:

Group 1 (S1), made up of aliquots to which were added a solution of 0.4 ml of UNC albumin at 20% and 0.6 ml of the mixture of 1.4 ml of 99.9% Sigma® dimethyl sulfoxide (DMSO), ID 2,489,421–4, and 2.6 mL of 0.9% sodium chloride, achieving a total concentration of 5% DMSO in each aliquot.

Group 2 (S2), made up of aliquots to which were added a solution of 1 ml of the mixture of 0.8 ml of 99.9% Sigma® dimethyl sulfoxide (DMSO), ID 2,489,421–4, and 3.2 ml of chloride sodium 0.9%, achieving a total concentration of 5% DMSO in each aliquot.

Group 3 (S3), made up of aliquots to which were added a solution of 1 ml of the mixture of 0.8 ml of 99.9% Sigma® dimethyl sulfoxide (DMSO), ID 2,489,421–4, and 3.2 ml of 5% dextrose, achieving a total concentration of 5% DMSO in each aliquot.

All aliquots in groups 1, 2 and 3 were subsequently subjected to rapid freezing at −80 °C. Five platelet aliquots from each group were randomly selected to be thawed in a 37 °C heat bath for 15 min and analyzed at 7, 14, and 28 days, respectively.

A group of 3-ml aliquots, now called “Control Group”, was kept at 22 °C under constant agitation for 5 days and used as a reference for the comparison of results.

Randomization and masking

The selection of the participating subjects was carried out by randomly choosing the medical history code registered in the hospital donor base. Once the donor samples were chosen, they were extracted and labeled by a member of the working group and then delivered to another member of the research team who was not aware of any identity data of the subjects and subsequently assigned the samples to the different groups by the simple randomization system, using the OxMaR software methodology.

After thawing, the samples were sent to another member of the team, who was unaware of the identity data or assignment group of the samples, to process them and perform tests to measure viability and functionality.

Survival and viability

Survival was assessed by the platelet count performed by an automatic hematology counter (Sysmex XN-550 Toa Medical). The measurement of the pH of the cryopreserved platelets after being thawed (expected values between 6.4 and 7.4) and the immunophenotyping of the platelets by flow cytometry (FC) FACS-Canto II mark, using Becton Dickinson Pharmingen™ CD41 (Gp IIb) and CD61 mouse-anti human (GP IIIa) conjugated murine fluorescent antibodies fluorescein 5 isothiocyanate (FITC), as well as CD42a mouse-anti human (Gp IX) picoerythrin (PE) V IP-34. The viability was evaluated by means of the difference (expressed in percentages) obtained between the number of platelets that marked negatively and positively for trypan blue staining, using the Neubauer chamber as an observation instrument.

Functionality

The functionality was evaluated from the percentage of platelet aggregation obtained after the stimulation of the preserved product with thrombin according to the following methodology:

500 μL of each aliquot of platelets were washed with pH 7.4 phosphate buffered saline (PBS) until a concentration of 1:10 was obtained and stimulated with 1 nM bovine thrombin. The reading was performed using a Chromo-Log model 490 platelet aggregometer at an optical density of 620 nm.

Approval by ethics committee

This study was approved by the institutional scientific review committee (Approval code: 1021), guaranteeing the compliance with international standards for research on biological samples and protection of the identity of the participants.

Results

During August 2021 and January 2022, 72 donors were recruited to enter the study, 79% men with a mean age of 31.6 ± 12 years and 21% women with a mean age of 52 ± 2 years. A total of 18 aliquots was assigned to the control group; the mean platelet count was 1934 ± 0.5 × 10^9/L, with a viability of 100%; all of them had a pH of 7.0 and a mean platelet volume of 11 ± 0.1 fl. A total of 99% of the platelets was positive for CD41, 98.5%, for CD61, and 96.5%, for CD42 (Table 1).

The aggregation percentage was 99%, starting its activity 30 s after being stimulated with thrombin. The rest of the aliquots collected were separated into sets of 18 units and assigned to groups 1, 2 and 3, whose analysis over time is described below (Table 1).

Day 7 post-freeze

Eighteen total aliquots (6 aliquots for each group) were analyzed.

In group 1, the mean platelet count after being thawed was 1844 ± 102 × 10^9/L with a viability of 98%; all aliquots reported a pH of 7.0 and the mean platelet volume (MPV) was 12.4 ± 1.4 fl. A total of 97.3% was positive for CD41, 96.7%, for CD61, and 93.2%, for CD42. The aggregation percentage was 90%, starting its activity 3 s after being stimulated with thrombin.

In group 2, the mean post-freezing platelet count was 1856 ± 76 × 10^9/L, with a viability of 96%; all aliquots reported a pH of 7.0 and the MPV was 15 ± 0.1 fl. A total of 98.4% was positive for CD41, 97.2% for CD61 and 91% for CD42. The aggregation percentage was 98%, starting its activity 2 s after being stimulated with thrombin.

In group 3, the post-freezing platelet count was 1752 ± 226 × 109/L, with a viability of 95%; all aliquots reported a pH of 7.0 and the MPV was 13.6 ± 0.3 fl. A total of 98% was positive for CD41, 98.3%, for CD61, and 92.1%, for CD42. The aggregation percentage was 89%, starting its activity 2 s after being stimulated with thrombin.

Day 14 post-freeze

Eighteen aliquots, distributed equally between groups 1 to 3, were analyzed. In group 1, the post-freezing platelet count was 1722 ± 238 × 109/L, with a viability of 96%; the average pH was 7.0 ± 0.1 and the MPV was 12 ± 1.3 fl. A total of 98.2% was positive for CD41, 98.3%, for CD61, and 96%, for CD42. The aggregation percentage was 88%, starting its activity 3 s after being stimulated with thrombin.

In group 2, the mean post-freezing platelet count was 1649 ± 215 × 109/L, with a viability of 95%; the average pH was 7.0 ± 0.1 and the MPV was 15 ± 0.1 fl. A total of 98.3% was positive for CD41, 98.1%, for CD61, and 92.4%, for CD42. The aggregation percentage was 98%, starting its activity 3 s after being stimulated with thrombin.

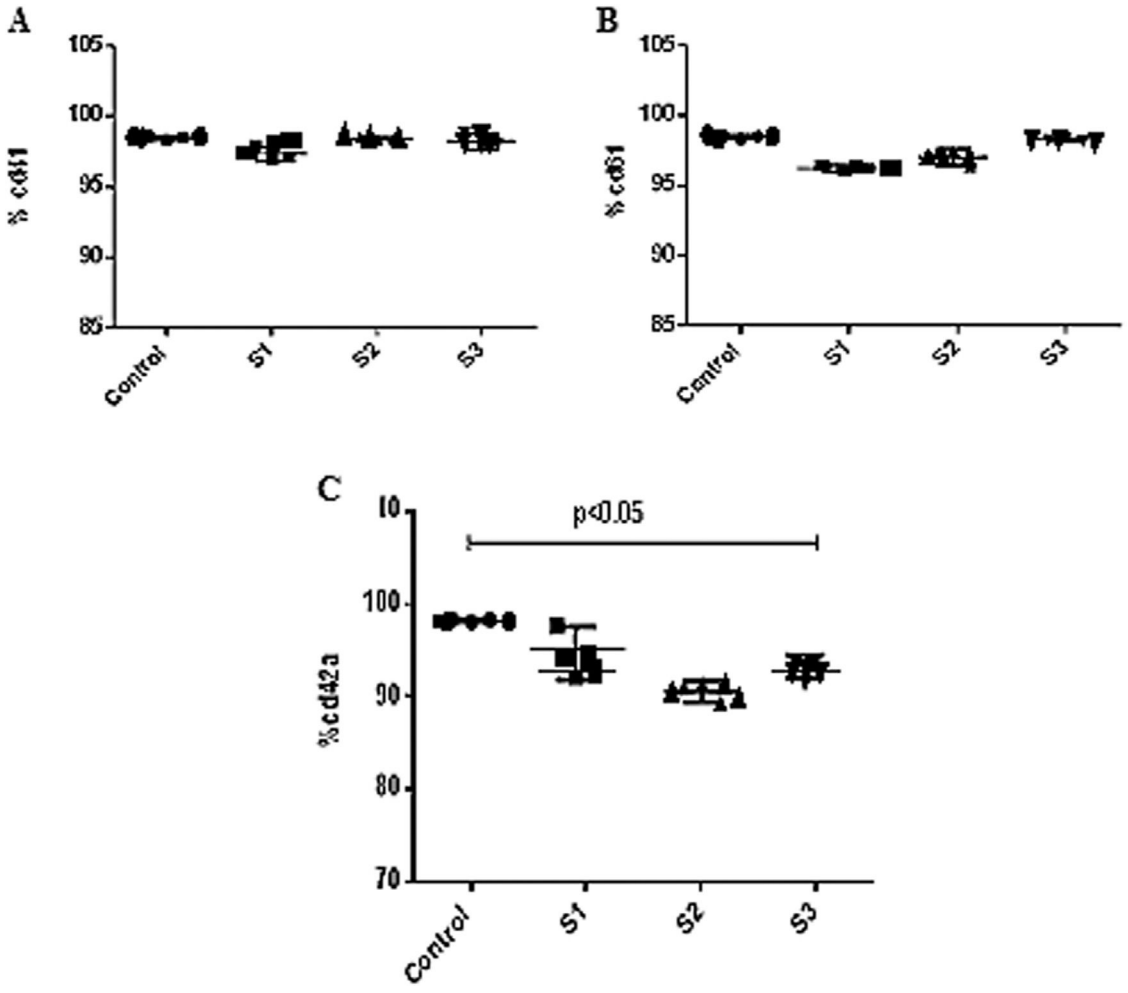
In group 3, the post-freezing platelet count was 1578 ± 223 × 109/L, with a viability of 94%; all aliquots reported a pH of 7.0 and the MPV was 12.9 ± 0.1 fl. A total of 94.7% was positive for CD41, 94.3%, for CD61, and 91.4%, for CD42. The aggregation percentage was 87%, starting its activity 3 s after being stimulated with thrombin.

Day 28 post-freeze

Eighteen aliquots, distributed equally between groups 1 to 3, were analyzed. In group 1, the mean post-freezing platelet count was 1602 ± 374 × 109/L, with a viability of 96%, average pH of 7.0 ± 0.1 and MVP of 14 ± 0.05 fl. A total of 94.8% was positive for CD41, 95%, for CD61, and 88.3%, for CD42. The

Table 1 – Platelet count, viability, labeling levels for membrane antigens and thrombin stimulation in cryopreserved platelets of Groups (Gr): 1(S1), 2 (S2) and 3 (S3).

Gr	Platelet count 10^9/L	Viability (%)	CD41 (%)	CD61 (%)	CD42a (%)	Aggregation percentage
	Day 7 Post-freeze					
S 1	1844 ± 102	98.0	97.3	96.7	93.2	90.0
S 2	1856 ± 76	95.0	98.4	97.2	91.0	98.0
S 3	1752 ± 226	94.0	98.0	98.3	92.1	89.0
	Day 14 Post-freeze					
S 1	1722 ± 238	96.0	98.2	98.3	96.0	88.0
S 2	1649 ± 215	95.0	98.3	98.1	92.4	98.0
S 3	1578 ± 223	94.0	94.7	94.3	91.4	87.0
	Day 28 Post-freeze					
S 1	1602 ± 374	96.0	95.0	94.8	88.0	84.0
S 2	1438,6 ± 429	94.0	94.0	95.0	91.8	95.0
S 3	1406,6 ± 436	93.0	94.0	92.0	97.8	82.0



Graph 1 – Comparison between Groups on Day 7 post-freezing.

aggregation percentage was 84%, starting its activity at 3 s of stimulation with thrombin.

In group 2, the platelet count was 1438.6 ± 429 × 109/L, with a viability of 94%, average pH of 7.0 ± 0.1 and VPM of 15 ± 0.05 fl. A total of 94.1% was positive for CD41, 94.6% for CD61 and 92% for CD42. The aggregation percentage was 95%, starting its activity 3 s after being stimulated with thrombin.

In group 3, the platelet count was 1406.6 ± 436 × 109/L, with 93% viability, average pH of 7.0 ± 0.1 and MVP of 13.9 ± 0.05 fl. A total of 94% was positive for CD41, 92.5%, for CD61, and 98.1%, for CD42. The aggregation percentage was 82%, starting its activity 3 s after being stimulated with thrombin.

Discussion

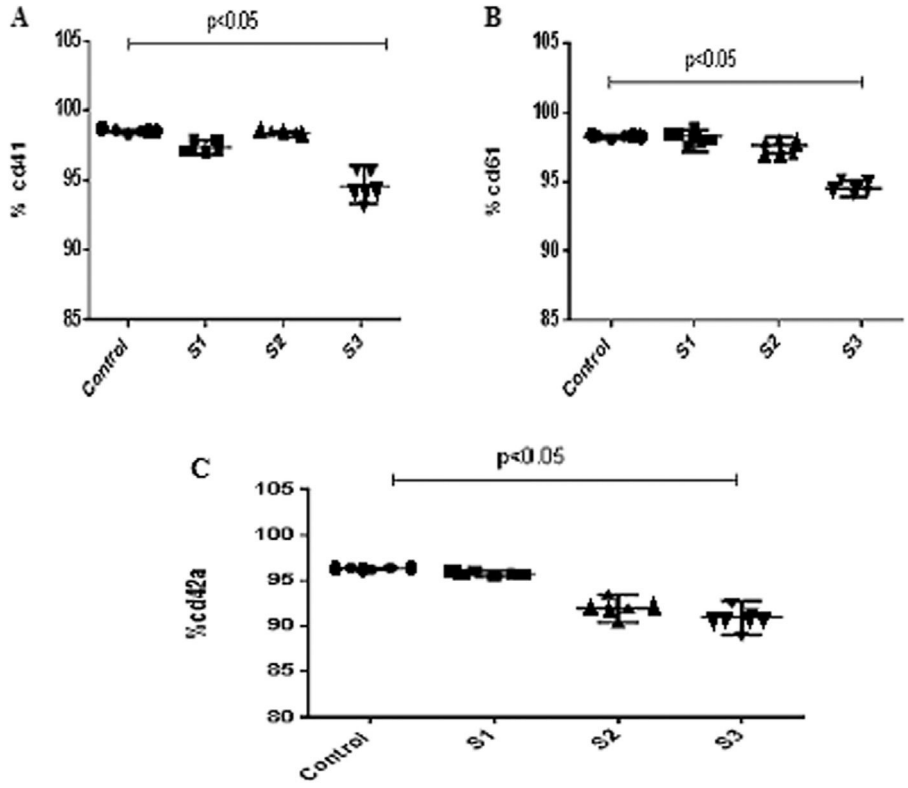
Using Dunn's multiple comparison test, with a 95% confidence interval, no statistically significant difference was reported in the mean platelet count of the control group and that obtained on day 7 post-freezing in groups 1, 2 and 3; likewise, no significant differences were obtained when internally comparing the values reported by these latter groups. The results of the platelet count obtained on days 15 and 28 post-freezing maintained a similar trend to that reported on day 7, not showing significant differences, compared to the control group, or in the comparison between groups.

On the other hand, no significant differences were observed in CD41, CD61 and CD42 marking between the control group and group 1 at day 7 post-freezing (95% CI Dunn's test); however, when comparing the same variables in group 2, a significant difference of 5.3% was reported in the percentage of marking for CD42 ($p < 0.05$). In group 3, the difference between the percentage of marking for CD42a, with respect to the control group, was 4.2%, this being statistically significant ($p < 0.05$) (Graph 1).

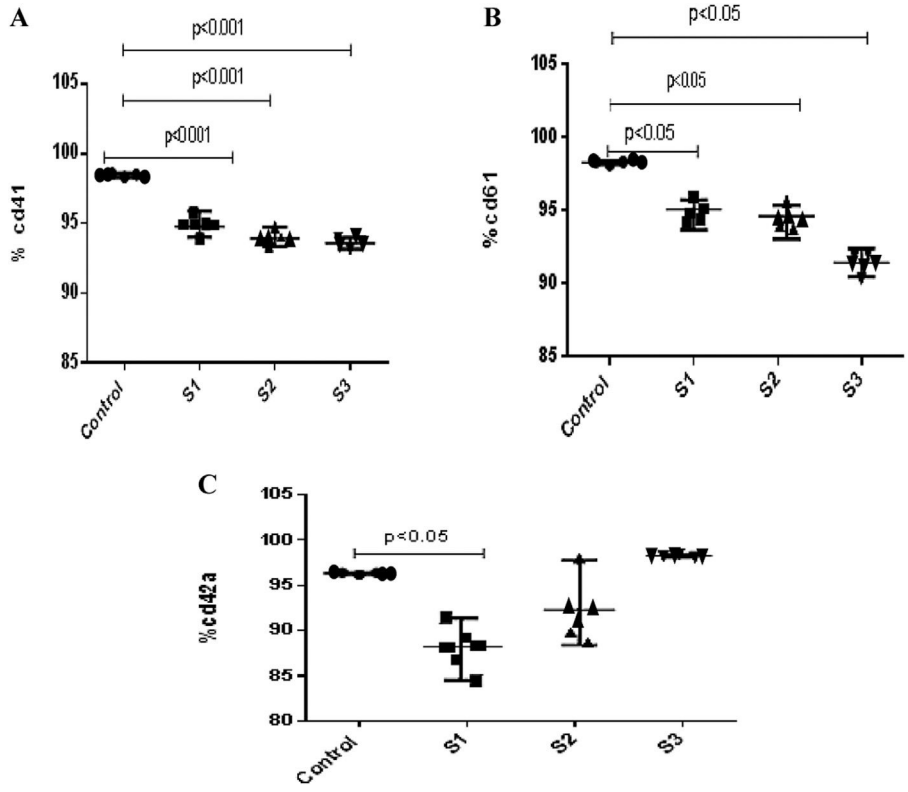
On day 14 post-freezing, significant differences were reported between the percentage of CD41 and CD61 staining in group 3, compared to the control group, of 5.3 and 3.1% ($p < 0.05$), respectively.

There was statistical significance in the percentage difference for CD42a marking in groups 2 and 3, compared to the control group (3.9 and 4.9%, respectively, with $p < 0.05$); this difference was also observed in comparison to the values obtained in group 1 (3.6 and 4.6%, respectively, with $p < 0.05$).

There were no statistically significant differences in the percentage of marking for CD41 and CD61 between groups 1 and 2 (Graph 2).



Graph 2 – Comparison between Groups on Day 14 post-freezing.



Graph 3 – Comparison between Groups on Day 28 post-freezing.

At day 28 post-freezing, the marking percentages reported significant differences for the 3 groups, compared to the control group. Regarding group 1, the difference was 3.6% ($p < 0.001$) in the CD41 marker, 3.2% ($p < 0.05$) in the CD61 marker and 8% ($p < 0.05$) in the CD42 marking. In group 2, the difference was 4.3% ($p < 0.001$) in the CD41 marker and 3.6% ($p < 0.05$) in the CD61 marker; no significant difference was reported in the percentage of CD42 labeling, compared to the controls. Group 3 presented a difference with the control group of 4.4% ($p < 0.001$) in the CD41 marker and 5.7% ($p < 0.05$) in the CD61 marker, with no significant difference in the CD42 marker (Graph 3). When comparing groups 1, 2 and 3, statistically significant differences were obtained in the percentage of marking for CD61 between groups 1 and 3 (2.8%, with a p-value of 0.02), as well as in the percentage of marking for CD42 between groups 3 and 1 (9.8%, with a p-value of 0.02).

Conclusions

The results of this research show that all frozen platelets, regardless of the solution used for their preservation, had a viability greater than 50%, mainly those associated with group 1, that presented higher percentages of viability, along with those of groups 1 and 2, which also reported higher levels of functionality. Likewise, the possibility of prolonging the survival time of stored platelets for up to 28 days, preserving levels of viability and functionality suitable for use in transfusion medicine, has been demonstrated.

Although the existence of other investigations that address platelet cryopreservation on small and medium scales in terms of survival and options for its use in real clinical scenarios has been documented, the present study stands out in two fundamental aspects:

In the first place, although the freezing method reported by Valeri et al. was taken as a reference, in our research we studied 2 different cryopreservative solution alternatives (solutions S1 and S3) to the conventional strategy of 5% DMSO in sodium chloride solution; our study made use of sodium in solution S2.

Secondly, this study was able to show in the same test the survival evaluation of cryopreserved platelets once they were removed from the freezer and thawed at room temperature, as well as their functionality in vitro through the study of platelet aggregation with thrombin.

Additionally, this research is highlighted as the first of its kind in Latin America and the Caribbean, this being a point of reference for conducting other research in Latin America and other regions of the world that consolidates the evidence described here.

Conflicts of interest

The authors reported in the following investigation declare that they have no conflicts of interest.

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