Calcium chelation: A novel approach to reduce cryopreservation-induced damage to frozen platelets

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Abstract

Background: Cryopreserved platelets are phenotypically and functionally different to conventionally stored platelets. Calcium may be released from internal stores during the freeze/thaw process, initiating signalling events which lead to these alterations. It was hypothesised that the addition of a calcium chelator prior to cryopreservation may mitigate some of these changes.

Methods: Buffy-coat derived platelets that had been pooled and split were tested fresh and following cryopreservation (n=8 per group). Platelets were cryopreserved using 5-6 % dimethylsulfoxide (DMSO) or were supplemented with increasing concentrations of the internal calcium chelator, BAPTA-AM (100 µM, 200 µM, or 400 µM), prior to storage at -80 °C.

Results: Supplementation of platelets with BAPTA-AM prior to freezing improved platelet recovery in a dose response manner (400 µM: 84 ± 2%) compared to standard DMSO cryopreserved platelets (70 ± 4%). There was a loss of GPIbα, GPVI, and GPIIb/IIIa receptors on platelets following cryopreservation, which was rescued when platelets were supplemented with BAPTA-AM (400 µM: $p<0.0001$ for all). Platelet activation markers, such as phosphatidylserine and P-selectin, were externalised on platelets following cryopreservation. However, the addition of BAPTA-AM significantly reduced the increase of these activation markers on cryopreserved platelets (400 µM: $p<0.0001$ for both). Both cryopreserved platelet groups exhibited similar functionality as assessed by thromboelastography, forming clots at a faster rate than fresh platelets.

Conclusions: This study demonstrates that calcium plays a crucial role in mediating cryopreservation-induced damage to frozen platelets. The addition of the calcium chelator, BAPTA-AM, prior to cryopreservation reduces this damage.

Keywords: platelet, cryopreservation, DMSO, calcium chelation, BAPTA-AM

Introduction

Conventional room-temperature storage of platelet concentrates in gas permeable bags with constant agitation limits the shelf-life to between five and seven days.[1] This results in logistical issues for supplying and maintaining adequate platelet stocks, particularly in remote and rural regions.[2] As such, the minimum requirements of clinical care may not be available in these areas.

The cryopreservation of platelets offers unique advantages over conventional storage techniques, extending the platelet shelf-life up to two years.[3] Platelet cryopreservation involves the addition of a cryoprotectant, dimethylsulfoxide (DMSO), and freezing at -80°C.[4,5] The phenotype and functionality of thawed cryopreserved platelets differs significantly to conventionally stored platelets.[6-8] These alterations include a loss of functional platelet surface receptors, externalisation of phosphatidylserine, shedding of microparticles and granule release.[6,8-10] It has also previously been demonstrated that cryopreserved platelets exhibit a higher basal calcium level than conventionally stored platelets,[8] which given the importance of calcium signalling in mediating platelet activation,[11,12] may contribute to the altered phenotype. Higher basal calcium levels have also been observed as a result of chilling platelets to 5 °C.[13]

In fresh platelets, calcium is responsible for regulating a wide range of cellular processes and is critical for platelet activation in haemostasis.[12] Calcium is released from internal stores within the dense tubular system or taken up from the external environment via calcium influx through the plasma membrane.[12] When conventionally stored platelets are stimulated with agonists in the presence of a calcium chelator, markers of platelet activation, including phosphatidylserine externalisation, granule release, and aggregation, are reduced.[14-16] While the role of calcium in mediating the altered phenotype of cryopreserved platelets has not been explored, we hypothesised that the cryopreservation process induces calcium mobilisation, which then

promotes the typical procoagulant phenotype. Therefore, the aim of this study was to characterise the role of calcium in mediating cryopreservation-induced damage using a calcium chelator. This was carried out by measuring the recovery, phenotype, and functionality of cryopreserved platelets frozen in the presence of an internal calcium chelator (BAPTA-AM) compared to the standard DMSO-only cryopreserved platelets (DMSO control).

Methods

Study design

Ethics approval was obtained from the Australian Red Cross Lifeblood (formerly the Australian Red Cross Blood Service; herein referred to as Lifeblood) Research Ethics Committee. All donations were collected from eligible, voluntary donors, in accordance with Lifeblood standard operating procedures.

Buffy coat-derived platelet units were prepared in 30 % plasma/70 % platelet additive solution (SPP+; Macopharma, Mouvaux, France), as previously described.[17] On day 1 post-collection, two ABO-matched platelet units were pooled and split to form matched pairs (n=8; Supplementary Figure 1). A sample was removed on day 1 from the platelet pool via sterile transfer for baseline testing (fresh).

Cryopreservation and thawing

For cryopreservation, 100 mL of 27 % wt/vol DMSO/0.9 % saline (Sypharma Pty. Ltd, Dandenong, VIC, Australia) was added to each platelet unit to achieve a final concentration of 5-6 % (v/v).[5] Subsequently, each platelet unit was split in half and transferred into 450 mL polyvinylchloride platelet storage bags (Macopharma) to create four individual units. All platelet units were then centrifuged to pellet the platelets and excess DMSO-containing supernatant was removed. Each platelet unit was randomly assigned to one of the four study arms: DMSO only (vehicle control), or containing different concentrations of the internal calcium chelator BAPTA-AM (1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester); Abcam, Cambridge, United Kingdom). The DMSO vehicle control or BAPTA-AM (final concentrations of 100 µM, 200 µM, or 400 µM) was diluted in a small volume of the DMSO-containing supernatant, which was then sterilely added to the assigned platelet unit. All platelet units were resuspended at a final volume of 12.5 mL, which is half the volume of a standard cryopreserved platelet hyperconcentrate.[18] The platelets were

then packaged and frozen at -80 °C, where they were stored for a minimum of one week prior to thawing.

Platelets were thawed in a water bath at 37 °C for 4-5 minutes before being rested on a platelet agitator (Helmer Inc., Noblesville, IN, USA) for 15 minutes.[19] Concurrently, two units of frozen whole blood derived plasma (FFP) were thawed in a water bath at 37 °C, before being pooled and split to create four matched units of approximately 120 mL. Following the resting period, platelets were resuspended in the freshly thawed plasma. All platelet units were visually inspected for the presence of swirl at the time of sampling. Platelet samples were taken from each unit immediately after resuspension.

Laboratory analysis

The platelet count and mean platelet volume (MPV) was determined using a haematology analyser (CELL DYN Ruby, Abbott Diagnostics, IL, USA). The platelet count was used to calculate the platelet content, platelet recovery following cryopreservation, and to normalise platelet counts in subsequent laboratory assays. The pH was measured at room temperature (Seven Excellence Multiparameter; Mettler Toledo, OH, USA).

The phenotype of platelets was characterised by flow cytometry using previously described protocols.[17] Platelets were diluted to 300 x 10^9 cells/L in Tyrode's buffer and were stained with the following antibodies: CD41a-PE, CD42b-PE, CD61-FITC, CD62P-PE, PAC-1-FITC (all obtained from BD biosciences, CA, USA), GPVI-efluor660 (Biolegend, CA, USA), or lactadherin-FITC (Haematologic Technologies Inc., VT, USA). Platelets were stained in the dark at room temperature for approximately 20 minutes before being further diluted with 1 mL of Tyrode's buffer and measured by flow cytometry. The percentage of positive cells was recorded, with 10,000 events being collected.

The basal calcium level was measured using the calcium indicator Fluo-3AM (Biotium, Inc., CA, USA), as previously described.[8] Briefly, platelets were diluted to 10 x 10^9 cells/L in Tyrode's buffer and loaded with Fluo-3AM and incubated in the dark at 37 °C for 15 minutes. The platelets were measured by flow cytometry without further dilution and the fluorescence was recorded for 30 seconds.

The absolute number of microparticles was determined by flow cytometry using TruCount tubes (BD biosciences), as previously described.[10] Briefly, platelet microparticles were identified as staining positive for CD61-APC (Dako, CA, USA) and Annexin-V-FITC (BioLegend), with gating parameters set to identify events ≤1.0 µm.

Platelet supernatant was collected following centrifugation. Briefly, platelets were centrifuged at 1600 *x* g for 20 minutes at room temperature and the supernatant was transferred to a new tube. The supernatant was subsequently centrifuged at 12,000 *x* g for 5 minutes at room temperature. The supernatant was transferred to a new tube and stored at -80 °C until testing.

The concentration of soluble CD62P (sP-selectin) was measured from the platelet supernatant according to the manufacturer's instructions (R&D systems, MN, USA). Absorbance at 450 nm was measured using a plate reader (VARIOSKAN LUX platform, Version 5.0.0.42, Thermo Scientific, Waltham, MA, USA), with samples being tested in triplicate against a standard curve.

The concentration of calcium in the platelet supernatant and plasma used to resuspend cryopreserved platelets was measured using a calcium detection assay kit, according to the manufacturer's instructions (Abcam). Absorbance at 575 nm was measured using a plate reader (VARIOSKAN LUX platform), with samples being tested in duplicate against a standard curve.

The platelet clotting potential was measured using a thromboelastogram (TEG 5000; Haemoscope Corporation, Niles, IL, USA), as previously reported.[20] Platelets were diluted to 200 x 10^9 cells/L in freshly thawed plasma and activated with kaolin and calcium chloride prior to application of sample to a plain cup. The samples were run for approximately 60 minutes at 37 °C and the following variables were recorded: R-time (time to clot initiation; min), K-time (speed of clot formation; min), maximum amplitude (MA; clot strength; mm), and α-angle (clot growth; degrees).

The procoagulant activity of platelets and platelet supernatant was assessed using a procoagulant phospholipid kit (STA-Procoag-PPL kit, Diagnostica Stago Ltd, Asnieres, France) according to the manufacturer's instructions. A sample (25 µL) of either platelets (100 x 10^6 cells/mL in Owren-Koller buffer; Diagnostica Stago Ltd) or platelet supernatant (neat) was used in the assay. Clot formation was analysed using an automated coagulometer (STACompact, Diagnostica Stago Ltd) and measured in seconds.

Statistical analysis

The data were analysed using GraphPad Prism 8.1.2 (GraphPad Software Inc.; La Jolla, CA, USA) and results are expressed as mean + standard deviation (SD). One-way analysis of variance (ANOVA) with repeated measures and *post-hoc* Bonferroni multiple comparisons test was used to assess differences between fresh and cryopreserved platelets and between DMSO only and cryopreserved platelets supplemented with BAPTA-AM. Linear regression with Pearson's correlation (r value) was performed to assess relationships between parameters. A p-value of less than 0.01 was considered to be significant.

Results

Following thawing and resuspension in plasma, the platelet content was significantly reduced in all cryopreserved units compared to fresh platelet units. Platelet recovery was 69.8 % in DMSO cryopreserved platelets, which significantly improved in a dose-dependent manner with all concentrations of BAPTA-AM tested (Table 1). Cryopreserved platelets had a higher pH compared to fresh platelets due to resuspension in plasma. However, there was no difference between the cryopreserved platelet groups (Table 1).

To confirm the relative abundance of calcium in cryopreserved platelets, both intracellular and extracellular calcium levels were assessed. Fresh and cryopreserved platelets were loaded with the calcium stain Fluo-3AM to assess intracellular calcium. The mean fluorescence of DMSO only cryopreserved platelets loaded with Fluo-3AM was approximately three-fold higher than fresh platelets (Figure 1A), and representative density scatterplots highlight this difference (Figure 1B). The concentration of calcium present in the supernatant of fresh platelets was approximately 0.5 mM, which increased four-fold following cryopreservation due to being resuspended in plasma (Figure 1C).

Alterations to the morphology of platelets was assessed following cryopreservation. Platelet swirl was present in fresh platelets prior to freezing, while little to no swirl was observed in DMSO cryopreserved platelets immediately following thawing (Table 1). Interestingly, platelets treated with BAPTA-AM displayed swirl immediately following thawing and resuspension, and the swirl observed with the highest concentration of BAPTA-AM was comparable to the swirling of the platelets prior to freezing (fresh). Fresh platelets had the highest MPV, which was reduced following cryopreservation (Figure 2A). Platelets treated with BAPTA-AM had a higher MPV than DMSO cryopreserved platelets, with the degree of improvement occurring in a dose-dependent manner, such that no difference was observed between the MPV of cryopreserved platelets supplemented with 400 µM of BAPTA-AM and fresh platelets (Figure

2A). A scatterplot of the forward scatter (FSC) and side scatter (SSC) profiles revealed differences in the light scattering properties of the platelets from each group (Figure 2B). Fresh platelets exhibited the highest FSC and SSC, while DMSO cryopreserved platelets exhibited the lowest FSC and SSC. Cryopreserved platelets supplemented with BAPTA-AM were distributed between these two populations, with a dose-dependent improvement in FSC and SSC properties, trending towards a return to that observed with fresh platelets (Figure 2B).

The platelet phenotype was characterised by flow cytometry. The percentage of platelets with surface expression of CD61 was high on fresh and cryopreserved platelets, with no difference observed between groups following thawing (data not shown). The percentage of fresh platelets with surface expression of GPIbα (CD42b), GPVI, and GPIIb (CD41a) was high (Figure 3A-C). Following cryopreservation, there was a reduction in the percentage of platelets staining positive for these markers. Notably, retention of these receptors was significantly increased in cryopreserved platelets supplemented with BAPTA-AM compared to the DMSO cryopreserved platelets. The percentage of fresh platelets expressing activated GPIIb/IIIa (measured using PAC-1), phosphatidylserine (measured using lactadherin), and P-selectin (CD62P) was low, but increased significantly following cryopreservation (Figure 3D-F). BAPTA-AM reduced the percentage of platelets expressing these markers, although the proportion of positive staining platelets was still higher than observed in the fresh units.

The size of platelets can influence the platelet phenotype, such that larger platelets may express more surface receptors to support platelet adhesion while smaller platelets shed surface receptors and externalise phosphatidylserine to become procoagulant.[21] To determine if these relationships were present in cryopreserved units, multiple parameters were compared. A strong correlation was found between the FSC of platelets and the expression of GPIbα, GPVI, and GPIIb, where a high FSC profile was associated with a greater percentage of platelets expressing these receptors (Figure 4A-C). In contrast, there was a strong inverse correlation between phosphatidylserine externalisation and the FSC profile of

the platelets (Figure 4D). There was also a strong inverse correlation between phosphatidylserine externalisation and GPIbα expression (Figure 4E), where cryopreserved platelets supplemented with BAPTA-AM demonstrated less phosphatidylserine externalisation and higher GPIbα expression than DMSO cryopreserved platelets. A similar correlation was observed with phosphatidylserine externalisation and GPVI expression (Figure 4F).

The platelet supernatant was analysed to determine the release of granular stores and microparticles. The supernatant from fresh platelets contained the lowest concentration of sP-selectin, which increased three-fold following cryopreservation (Figure 5A). The concentration of sP-selectin in the supernatant of platelets treated with BAPTA-AM remained high compared to fresh platelets but was reduced compared to DMSO cryopreserved platelets. There was a strong correlation between surface and soluble P-selectin (Figure 5B), where fewer BAPTA-AM supplemented cryopreserved platelets expressed P-selectin and a lower concentration of sP-selectin was present in the platelet supernatant compared to DMSO cryopreserved platelets. The absolute number of microparticles was low in fresh platelet units (Figure 5C). Following cryopreservation, the number of microparticles shed was increased approximately 100-fold compared to fresh platelets (Figure 5C). BAPTA-AM was unable to prevent microparticle formation, with no difference in the number of microparticles shed between DMSO and BAPTA-AM cryopreserved platelets.

The functional impact of calcium chelation on the clot forming ability of platelets was measured. The time until clot formation (R-time) was approximately 11 minutes in fresh platelets (Figure 6A). This time was approximately halved in cryopreserved platelets, with no difference between DMSO cryopreserved platelets and those supplemented with BAPTA-AM. Fresh platelets formed stronger clots (maximum amplitude) than cryopreserved platelets (Figure 6B), and the addition of BAPTA-AM had no effect on this parameter. Fresh platelets had a longer K-time and smaller α-angle compared to cryopreserved platelets; however, no

differences were observed between the cryopreserved platelet groups (data not shown). Using a phospholipid dependent clotting assay, fresh platelets took 95 seconds to form a clot, which was reduced to approximately 20 seconds following cryopreservation (Figure 6C). No difference in clotting time was observed between DMSO cryopreserved platelets and cryopreserved platelets supplemented with BAPTA-AM. A similar trend was observed for the platelet supernatant clotting times (Figure 6D).

Discussion

This study examined the ability of the calcium chelator, BAPTA-AM, to mitigate cryopreservation-induced damage to frozen platelets. Cryopreservation dramatically alters the platelet phenotype compared to conventionally stored platelets. Specifically, markers associated with platelet activation are up-regulated.[6-8] As calcium is crucial for platelet activation, we hypothesised that it may be involved in mediating at least some of the changes observed in cryopreserved platelets. Here we demonstrate that the addition of an internal calcium chelator was able to reduce some of the damage undergone by platelets during the cryopreservation process, improving platelet recovery and phenotype, while preserving the typical functionality of cryopreserved platelets.

The recovery of DMSO platelets following cryopreservation was in line with previously reported results.[9,20,22] The addition of BAPTA-AM prior to freezing improved platelet recovery following cryopreservation. This may have been due to a reduction in damage during the freezing process. The presence of the calcium chelator within the cell may have increased the concentration of solutes, providing additional protection during the cryopreservation process.[23] Increasing the concentration of solutes within the product reduces the amount of ice that can form upon freezing.[23] However, this must be carefully balanced to avoid toxic side effects from excess salts, such as apoptosis.[23,24] The increased solute concentration achieved here appears to be beneficial, likely reducing cell shrinkage due to osmotic dehydration during the cryopreservation process,[23] enabling better preservation of the platelet size and structure. This is reflected by the improved cell recovery, MPV and light scattering profiles of platelets treated with BAPTA-AM. Further, the ability of BAPTA-AM supplemented platelets to swirl immediately following thawing and resuspension indicates that these platelets are discoid-shaped[1,25] and more structurally similar to fresh platelets than standard DMSO cryopreserved platelets. Importantly, the presence of platelet swirl has previously been correlated with *in vivo* viability after transfusion,[26] which may be beneficial for the transfusion of cryopreserved

platelet products. Overall, these results suggest that BAPTA-AM may have a cryoprotective effect when used in conjunction with the standard platelet cryoprotectant, DMSO.

The platelet surface receptors GPVI and GPIbα are highly susceptible to cryopreservation-induced damage, with significantly less cryopreserved platelets expressing these receptors compared to fresh platelets.[5,8,22,27] Calcium mobilisation during cryopreservation appears to play a role in the loss of these receptors, as calcium chelation prior to freezing was able to significantly reduce the loss of these receptors on platelets following thawing. The improved preservation of platelet size may also enable better retention of these receptors on BAPTA-AM treated platelets, with strong correlations found between the proportion of platelets expressing these receptors and forward scatter properties. The loss of these receptors on cryopreserved platelets may occur via proteolytic pathways leading to ectodomain shedding.[21,28-30] Platelet activation is known to result in metalloproteolytic shedding of the platelet receptors GPIbα and GPVI as a mechanism to regulate thrombus growth.[21,31,32] The increased intracellular calcium concentration in cryopreserved platelets appears sufficient to cause a similar result, initiating events that lead to the reduced surface expression of these receptors in the absence of agonist stimulation. Further, it has been identified that sub-populations of activated platelets which shed GPIbα and GPVI receptors, also have a high intracellular calcium concentration and high phosphatidylserine externalisation, which is characteristic of the change from adhesive to procoagulant platelet function.[21] These findings are aligned with the results presented here, as there was a strong negative correlation between phosphatidylserine externalisation and a loss of GPIbα or GPVI, reflective of the procoagulant phenotype of cryopreserved platelets.[33,34]

Phosphatidylserine externalisation and granule release are thought to occur in a calcium-dependent manner,[11,12,35] as loading platelets with an internal calcium chelator significantly reduces these activation markers when platelets are stimulated with potent agonists.[36,37] In contrast, phosphatidylserine externalisation during platelet apoptosis occurs independently of

changing intracellular calcium concentrations.[11,38] BAPTA-AM supplementation of cryopreserved platelets significantly reduced the externalisation of phosphatidylserine, indicating that phosphatidylserine externalisation during platelet cryopreservation is calcium dependent and thus may be the result of platelet activation rather than via the induction of apoptotic pathways. P-selectin is released from alpha granules during platelet activation and can either be expressed on the platelet membrane or shed from platelets and function in a soluble form.[35,39] Fewer BAPTA-AM supplemented cryopreserved platelets expressed surface P-selectin and the supernatant contained a lower concentration of soluble P-selectin compared to DMSO cryopreserved platelets, indicating that these platelets do not merely shed more P-selectin. Thus, BAPTA-AM supplemented cryopreserved platelets appear to better retain their alpha granules. Taken together, these results demonstrate that calcium chelation prior to cryopreservation reduces the typical activation markers expressed on cryopreserved platelets.

Platelet microparticles are shed during platelet storage and following platelet activation.[11,40,41] However, the number of platelet microparticles formed following stimulation with potent platelet agonists, such as collagen or thrombin-receptor activating peptide-6, is still significantly less than the number formed as a result of cryopreservation.[8] Thus, the mechanism driving the release of the microparticles is likely different. The results presented here demonstrate that microparticle formation during platelet cryopreservation occurs independently of calcium, thus differing from agonist induced microparticle release, which seems to be calcium dependent.[11,40] Microparticle formation during cryopreservation appears inherent to the process and may occur through membrane fragmentation rather than controlled release. The dramatic increase in microparticle content is likely driven by extensive cytoskeletal reorganisation,[42-44] which is amplified during cryopreservation,[10] leading to uncontrolled membrane fragmentation. This likely occurs as a result of membrane damage during both the freezing and thawing process. Importantly, the presence of microparticles following cryopreservation seems advantageous, providing essential support for the rapid clot

forming ability of cryopreserved platelet products,[20,45] with the rapid clotting time in the PPL assay driven largely by the microparticles in the platelet supernatant. Thus, reducing the shedding of microparticles may not actually be considered as an 'improvement' to a cryopreserved platelet product.

Despite being phenotypically different to conventionally stored platelets, *in vitro* evidence demonstrates that cryopreserved platelets remain functional and are capable of forming clots at a faster rate.[7,20,22] As phosphatidylserine is important for the procoagulant activity of cryopreserved platelets,[20] it was surprising that the reduction in phosphatidylserine externalisation on BAPTA-AM treated platelets did not result in a dampening of the functional output. Regardless of the reduced phosphatidylserine externalisation, cryopreserved platelets supplemented with BAPTA-AM remained highly procoagulant and formed clots at a similar rate to standard DMSO cryopreserved platelets. It is not yet known how much phosphatidylserine is required to maintain the functional advantage of cryopreserved platelets. The proportion of phosphatidylserine-expressing platelets in cryopreserved units supplemented with the highest concentration of BAPTA-AM was still 10-fold higher than fresh platelets. Further, as phosphatidylserine-expressing microparticles were still highly abundant in all cryopreserved platelet units, their contribution as a procoagulant mediator likely outweighs any reduction in phosphatidylserine-expressing platelets.

Despite the improved receptor retention on BAPTA-AM supplemented cryopreserved platelets, no enhancements to clot strength were observed. This may be reflective of the reduced ability of cryopreserved platelets to respond to agonist stimulation and upregulate activation markers or aggregate.[8] Further, the calcium chelator may remain active following cryopreservation, impacting the ability of platelets to amplify activation signals. A more sensitive assay or the investigation of downstream signalling pathways may be required in order to tease out the differences in functional capacities of cryopreserved platelets.[46-48]

Cryopreserved platelets can be resuspended in a variety of media, such as plasma or platelet additive solution.[8,9,49] The present study used 100% plasma to resuspend platelets, which contained approximately 1.8 mM calcium. While it was hypothesized that the calcium dependent cryopreservation-induced damage was occurring during freezing, it was not known if the increase in extracellular concentration of calcium upon resuspension would also contribute. To assess this, the quality of platelets supplemented with BAPTA-AM prior to freezing was compared to units where BAPTA-AM was added both prior to freezing and immediately after thawing but prior to resuspension. The addition of post-thaw BAPTA-AM did not result in further improvements to the platelet recovery or phenotype (data not shown). This suggests that the calcium-mediated damage occurred primarily during platelet freezing and not as a result of calcium influx during thawing. This is in line with previous studies that demonstrate that intracellular calcium is primarily released during platelet cooling to 5 °C but not during rewarming to 20 °C.[13]

This study focused on analysing the immediate post-thaw recovery, phenotype and functionality of platelets supplemented with up to 400 µM of BAPTA-AM prior to freezing. Our study design used half-sized platelet concentrates, reducing the platelet content and freezing volume to half that of a typical hyperconcentrated product, with the possibility of altering the dynamics of the freezing profile,[50] and thus the resulting post-thaw platelet quality. However, as the recovery, phenotype and functionality of DMSO cryopreserved platelets remained similar to previously reported results,[8,9] these modifications appear to have had minimal effect on the outcomes described here. An additional consideration is that the intended doses of BAPTA-AM were lower than targeted due to cleavage of acetoxymethyl (AM) by extracellular esterases, as reported in *in vivo* studies.[51] As such, given that as the protective effects afforded by BAPTA-AM are clearly dose dependent, it may be worthwhile investigating whether further improvements are possible with a higher concentration of BAPTA-AM.

In summary, this study has provided novel insight into the role calcium plays in mediating the altered phenotype of cryopreserved platelets. While the addition of calcium chelators provides critical information as to how cryopreserved platelets are altered by intracellular calcium, further research is required to establish the suitability of BAPTA-AM in a clinical setting in order to determine whether this would be a practical solution. Here we have demonstrated that treating platelets with BAPTA-AM prior to cryopreservation improves platelet recovery and quality. This is the first study to comprehensively analyse the impact of intracellular calcium chelation on cryopreserved platelet quality and function, and highlights calcium signalling as a potential target to improve the overall quality of cryopreserved platelet products. Interestingly, pre-freeze calcium chelation effectively impeded some calcium-dependent pathways but not others. Further characterisation of signalling events mediating the altered phenotype of cryopreserved platelets would be beneficial to broaden the understanding this specialised product.

Author contributions: LJ, LW, and DCM conceived and designed the study. LW and LJ performed the experiments, analyzed the data, and drafted the manuscript. All authors contributed to writing the paper, critically reviewed, and approved the manuscript for publication.

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Table 1. Specifications of platelet components before freezing and after thawing and resuspension in plasma

Parameter	Fresh	Cryopreserved			
		DMSO	BAPTA-AM 100μM	BAPTA-AM 200μM	BAPTA-AM 400μM
Recovery (%)	NA	69.8 ± 3.5	78.2 ± 2.2†	82.1 ± 2.5†	83.6 ± 2.4†
pH (20-24 °C)	7.0 ± 0.2	7.3 ± 0.3*	7.3 ± 0.3*	7.3 ± 0.2*	7.2 ± 0.4*
Swirl	Present	Absent	Present	Present	Present

Values are presented as mean ± standard deviation; n=8 in each group. *p<0.01 compared to fresh platelets. †p<0.01 compared to DMSO cryopreserved platelets. NA = not applicable.

Figure legends

Figure 1. Cryopreserved platelets contain a higher basal calcium level compared to fresh platelets

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). **(A)** Platelets were loaded with the calcium stain Fluo-3AM and median fluorescence intensity (MFI) was measured by flow cytometry for 30 seconds. **(B)** Representative density scatter plots of Fluo-3AM fluorescence. **(C)** The concentration of calcium in the platelet supernatant and plasma used to resuspend cryopreserved platelets was measured using a colorimetric calcium assay. Data represents mean + standard deviation (error bars). *$p<0.01$ compared to fresh.

Figure 2. Calcium chelation preserved platelet size and scatter properties following cryopreservation

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). For cryopreservation, platelets were either frozen with DMSO (DMSO only) or were supplemented with increasing concentrations of BAPTA-AM (100 µM, 200 µM, or 400 µM) before freezing and storage at -80 °C. Platelets were thawed, resuspended in plasma, and sampled for testing. **(A)** The mean platelet volume was measured using a haematology analyser. The scattering properties of platelets was measured by flow cytometry. **(B)** A scatterplot depicting the correlation between the forward and side scatter profiles of each sample. Data represents mean + standard deviation (error bars) or individual data points (scatterplot). *$p<0.01$ compared to fresh; †$p<0.01$ compared to DMSO cryopreserved platelets.

Figure 3. Calcium chelation improves receptor retention and reduces activation markers following cryopreservation

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). For cryopreservation, platelets were either frozen with DMSO (DMSO only) or were supplemented with increasing concentrations of BAPTA-AM (100 μM, 200 μM, or 400 μM) before freezing and storage at -80 °C. Platelets were thawed, resuspended in plasma, and sampled for testing. Platelets were stained with **(A)** CD42b-PE, **(B)** GPVI-eFluor660, **(C)** CD41a-PE, **(D)** PAC-1-FITC, **(E)** Lactadherin-FITC, or **(F)** CD62P-PE and the percentage of positive cells was measured by flow cytometry following the collection of 10,000 events. Data represents mean + standard deviation (error bars). *$p<0.01$ compared to fresh; †$p<0.01$ compared to DMSO cryopreserved platelets.

Figure 4. The relationships between platelet forward scatter properties, surface glycoprotein expression and phosphatidylserine externalisation

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). For cryopreservation, platelets were either frozen with DMSO (DMSO only) or were supplemented with increasing concentrations of BAPTA-AM (100 μM, 200 μM, or 400 μM) before freezing and storage at -80 °C. Platelets were thawed, resuspended in plasma, and sampled for testing. The forward scatter properties of platelets was measured by flow cytometry. In addition, platelets were stained with CD42b-PE, GPVI-eFluor660, CD41a-PE, or Lactadherin-FITC, and the percentage of positive cells was measured by flow cytometry. Scatterplots depicting the correlation between forward scatter and **(A)** GPIbα, **(B)** GPVI, **(C)** CD41a, or **(D)** Lactadherin (phosphatidylserine) and between Lactadherin (phosphatidylserine) and **(E)** GPIbα or **(F)** GPVI. Data are shown as individual data points.

Figure 5. Calcium chelation reduces P-selectin shedding but does not impact microparticle release following cryopreservation

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). For cryopreservation, platelets were either frozen with DMSO (DMSO only) or were supplemented with increasing concentrations of BAPTA-AM (100 µM, 200 µM, or 400 µM) before freezing and storage at -80 °C. Platelets were thawed, resuspended in plasma, and sampled for testing. The concentration of **(A)** soluble P-selectin (sCD62P) was measured from the platelet supernatant by ELISA. **(B) A scatterplot depicting the correlation between surface expressed P-selectin (CD62P) and sCD62P is shown. (C)** Platelet microparticles staining positive for CD61-APC and Annexin V-FITC were enumerated by flow cytometry. Data represents mean + standard deviation (error bars) **or as individual data points (scatterplot)**. *p<0.01 compared to fresh; †p<0.01 compared to DMSO cryopreserved platelets.

Figure 6. Calcium chelated cryopreserved platelets maintain rapid clot forming ability

Platelets were sampled before freezing (fresh) and following cryopreservation (cryopreserved). For cryopreservation, platelets were either frozen with DMSO (DMSO only) or were supplemented with increasing concentrations of BAPTA-AM (100 µM, 200 µM, or 400 µM) before freezing and storage at -80 °C. Platelets were thawed, resuspended in plasma, and sampled for testing. The clot forming ability of platelets was measured using thromboelastography (TEG). **(A)** The R-time (reaction time; time until clot formation) and **(B)** the maximum amplitude (maximum strength of the clot) was recorded. The clotting time of **(C)** platelets (100 x 10^6 cells/mL) and **(D)** platelet supernatant was measured with Procoag-PPL assay. Data represents mean + standard deviation (error bars). *p<0.01 compared to fresh platelets.

Supplementary Figure 1. Study design

On day 1 following collection, two ABO-matched buffy coat-derived platelet units were pooled and a sample (15 mL) was removed for baseline testing. The pool was equally split to create a matched pair. For cryopreservation, DMSO was added to each platelet unit to achieve a final concentration of 5-6 %. Following DMSO addition, each platelet concentrate was split in half to create four discrete units. All platelet units were centrifuged at 1350 x g for 10 minutes at room temperature. The majority of the DMSO-containing supernatant was then removed, creating a platelet pellet. BAPTA-AM was prepared in the DMSO-containing supernatant, to achieve a final concentration of 100 µM, 200 µM, or 400 µM, which was then sterilely added to the assigned platelet unit. All platelet units were resuspended to a final volume of 12.5 mL, which is half that of a full sized cryopreserved platelet concentrate. The platelets were then packaged before freezing at -80 °C. Platelet products were stored for a minimum of one week prior to thawing.