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In vitro Hemostatic Functions of Cold-Stored Platelets

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Keywords
Platelets · Cold storage · In vitro hemostatic functions

Abstract
Background: Transfusion of platelets is a life-saving medical strategy used worldwide to treat patients with thrombocytopenia as well as platelet function disorders. ***Summary:*** Until the end of 1960s, platelets were stored in the cold because of their superior hemostatic functionality. Cold storage of platelets was then abandoned due to better posttransfusion recovery and survival of room temperature (RT)-stored platelets, demonstrated by radioactive labeling studies. Based on these findings, RT became the standard condition to store platelets for clinical applications. Evidence shows that RT storage increases the risk of septic transfusion reactions associated with bacterial contamination. Therefore, the storage time is currently limited to 4–7 days, according to the national guidelines, causing a constant challenge to cover the clinical request. Despite the enormous efforts made to optimize storage conditions of platelets, the quality and efficacy of platelets still decrease during the short storage time at RT. In this context, during the last years, cold storage has seen a renaissance due to the better hemostatic functionality, reduced risk of bacterial contamination, and potentially longer storage time. ***Key Messages:*** In this review, we will focus on the impact of cold storage on the in vitro platelet functions as promising alternative storage temperature for future medical applications.

Introduction

Platelets are small anucleated blood cell fragments (2–4 μm), produced from polyploid cells, called megakaryocytes, which reside in the bone marrow [1]. Platelets are characterized by short half-life, 7–10 days in humans, after which they are cleared from the circulation through the spleen or liver [2, 3]. The primary role of platelets is maintaining the hemostasis in order to stop blood loss [4]. The activity of circulating platelets is regulated by a balance of activating and inhibiting factors. In fact, under physiological conditions, platelets remain in a quiescent status, while after vessel damage cell activation is triggered by several blood and vessel components. After injury, platelets are captured from the circulation to adhere to the damaged area (extracellular matrix) and become activated. Upon activation, platelets [5] release their granules content and recruit more platelets, which in turn aggregate to form a platelet "plug" (Fig. 1a–c) [6]. Finally, via interaction with activated plasmatic coagulation factors, platelets contribute to thrombus formation, which ends with the formation of a stabile blood clot to block blood loss [4].

Giving their hemostatic functions, transfusion of platelets is an essential medical therapy used for prophylactic and therapeutic treatments to prevent or stop bleeding, respectively [5, 7]. Historically, until the early 1960s, platelet concentrates (PCs) were stored in the cold and usually transfused few hours post-collection. However, due to the increasing demand of blood products the production was enhanced and the storage time had to be prolonged, beginning the challenging debate about the

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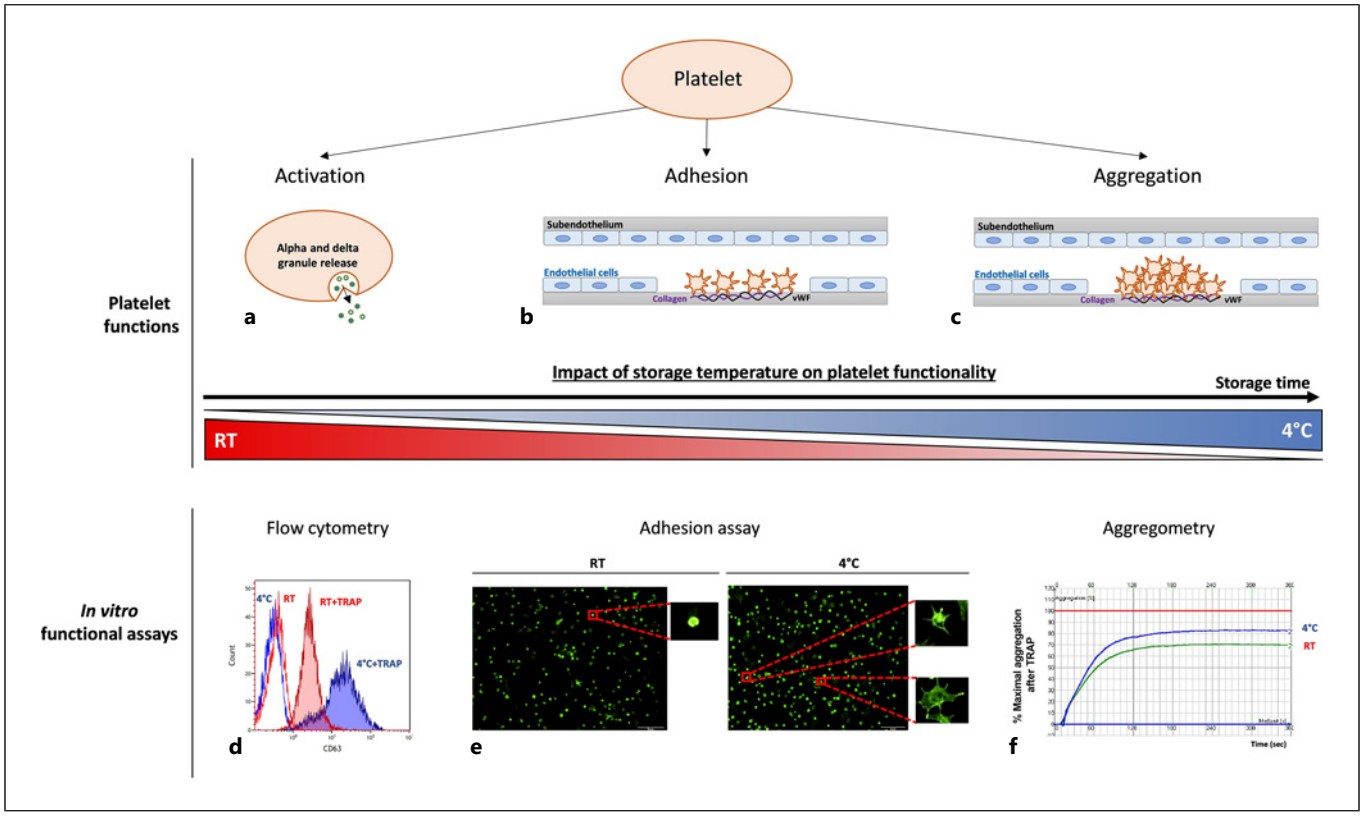

Fig. 1. Schematic representation of the different platelet functions and the corresponding in vitro assays. Upper panel: schematic illustration of (**a**) activation, (**b**) adhesion, and (**c**) aggregation abilities of the platelets. Lower panel: (**d**) representative overlay histograms of flow cytometric analysis reporting the CD63 expression of room temperature (RT, empty histogram: buffer) or 4°C stored (empty histogram, buffer) platelet concentrates, with and without TRAP stimulation. **e** Representative immunofluorescence pictures of adherent cells on a fibrinogen-coated surface after RT or 4°C storage, in response to TRAP (Green signal: calcein staining. Scale bar, 20 μm). **f** Representative aggregation curves of platelet concentrates, stored at RT or 4°C, measured after TRAP stimulation. vWF, von Willebrand factor.

optimal storage condition. In fact, clinical studies in the past reported better correction of bleeding time, in thrombocytopenic patients, upon transfusion with cold-stored platelets (CSPs), compared to room temperature (RT, 22–24°C) [8, 9]. However, in 1969 Murphy and Gardner [10] demonstrated that CSPs have significantly reduced half-life and posttransfusion recovery than RT-stored PCs. This led to a global change to RT storage, which is still the standard storage temperature of PCs used for clinical applications, despite the superior hemostatic functionality of CSPs demonstrated in some in vitro studies. Nevertheless, the debate is still open due to critical drawbacks of the RT storage. The latter is the ideal condition for bacterial growth which might lead to undesired side effects like posttransfusion septic reactions [11]. Therefore, in order to reduce this risk, the current storage time of PCs is limited to 4–7 days, the last only if pathogen inactivation technology is implemented which improves detection and prevention of bacterial contamination and therefore the safety of PCs but does not eliminate the residual and intrinsic risk of bacterial contamination completely [5, 12]. Beside the risk of bacterial growth, an additional reason for the short shelf life of RT-stored PCs is the accumulation of numerous biochemical, structural, and metabolic changes, collectively named "platelet storage lesions," which is storage time-dependent and causes a significant reduction of the platelet functionality during RT storage [3, 13]. In this context, alternative storage conditions have been intensively investigated and cold storage has seen something of a renaissance, during the last decade. In fact, it might reduce the risk of bacterial contamination, potentially extend the storage time, and preserve the platelet hemostatic functions. In the last years, several groups have reported better platelet function of CSPs, in terms of activation, adhesion, and aggregation, compared to RT-stored platelets [14–16]. Despite the superior cell functionality, the medical use of CSPs is still under debate due to two major limitations: reduced recovery and fast clearance in vivo, compared to RT storage [10, 16]. In this review, we will discuss in detail the current state of knowledge regarding the effects of cold storage on the in vitro hemostatic functions of platelets as promising alternative storage condition.

Standard Methods to Evaluate Platelet Functionality in vitro

Giving the key role of platelet transfusion in modern medicine, the analysis of the cell quality is extremely important to achieve best patient-related outcome. Therefore, several in vitro functional assays have been developed during the last years to evaluate platelet functions.

Flow cytometry is a widely used methodology, which allows qualitative and quantitative assessment of cell phenotype and functionality. For example, it assesses granule release and the resulting surface expression of activation markers, like CD62p or CD63, in response to different agonists (Fig. 1a, d). Moreover, analyses by flow cytometry allow investigation of intracellular transduction, detecting the expression of different proteins and their active/inactive status, such as the assessment of the phosphorylation status of the vasodilator-stimulated phosphoprotein (VASP), which plays an important role in the rearrangement of the actin cytoskeleton during platelet adhesion [17]. Additionally, biochemical intracellular changes can be analyzed, i.e., intracellular calcium concentration level or modification of the mitochondrial membrane potential. Flow cytometry has several advantages: small volume is required and whole blood samples, washed platelets, platelet-rich plasma, or PCs can be analyzed [18, 19]. On the other hand, certain expertise is needed for data analysis and in some cases for sample preparation.

Another well-established method, which is usually considered the gold standard to investigate platelet functionality in vitro, is the light transmission aggregometry (LTA) [20, 21]. LTA is a photometric-based assay and platelet aggregation is evaluated by measuring the changes in the transmission of light over time (Fig. 1c, f). Furthermore, by using different agonists, multiple receptors and pathways that regulate platelet aggregation can be tested. Therefore, LTA is widely used to diagnose platelet disorders and to monitor antiplatelet therapies [22]. The major advantages of these assays are that it is well-established, it allows analysis of the kinetics of platelet responses (including shape changes and reversible aggregation) and is relatively inexpensive. However, the process of sample preparation is time-consuming and potentially prone to errors [23].

While the flow cytometry and LTA can be used to assess the response to exogenous agonist and the ability of platelets to build cell aggregates, these assays cannot predict the ability of platelets to interact with extracellular matrix structure. The adhesion assay allows the assessment of platelet interaction with subendothelial proteins such as collagen and von Willebrand factor (Fig. 1b), which usually occurs in response to vessel wall damage [24]. In particular, the morphological changes, which characterize the different steps of the adhesion process, can be detected and analyzed by microscopy (Fig. 1e). Moreover, this assay can be performed either under static conditions or under shear stress [25]. Interestingly, using different coating surfaces (collagen, fibrinogen, etc.), a variety of signaling pathways can be assessed and this assay can be helpful for diagnostic purposes in selected patients. Nevertheless, since the performance and the data analysis are time-consuming and prone to pre-analytical errors, this assay is not routinely performed for the diagnosis of platelet disorders.

In addition, platelet functions and quality can be evaluated by the assessment of certain metabolic parameters, for example, pH as well as glucose and lactate levels. It has been reported that cold storage of platelets causes a decrease of the metabolic status indicated by a higher glucose and lower lactate concentration compared to platelets stored at RT [14, 26, 27]. Furthermore, another study showed that delayed-CSPs (5 storage days at RT under agitation followed by refrigeration without agitation until storage day 21) are characterized by enhanced in vitro functionality, in comparison to RT storage [28].

Impact of Cold Storage on in vitro Platelet Functions

CSP Activation

As already mentioned, during the last decades, numerous studies have reported better in vitro platelet functionality of CSPs compared to RT in a wide range of different assays [27, 29, 30]. For example, CSPs exhibit increased activation, indicated by the raise in the expression of activation markers, such as CD62p and CD63, upon stimulation with different agonists [16, 29]. These findings indicate enhanced platelet reactivity in CSPs. Moreover, it has been recently observed that even the basal expression of CD62p during cold storage of apheresis-derived PCs is significantly higher compared to RT, which can be explained by multiple factors like cold-induced activation of platelets during storage via cross linking glycoprotein Ib or increased interaction with protein complexes [15, 31, 32]. Additionally, enhanced formation of procoagulant platelets (defined by co-expression of CD62p and GSAO) in buffy coat-derived CSPs, without stimulation by agonists, was detected over the storage time of 15 days compared to RT-stored platelets and associated with higher peak thrombin, shorter lag time as well as shorter time to peak in the thrombin generation assay [33]. This indicates that during cold storage the presence of functional platelet subpopulations is increased in comparison to RT. Indeed, CSPs have been described as "primed" for the activation [34]. Consequently, concerns about the increased risk of

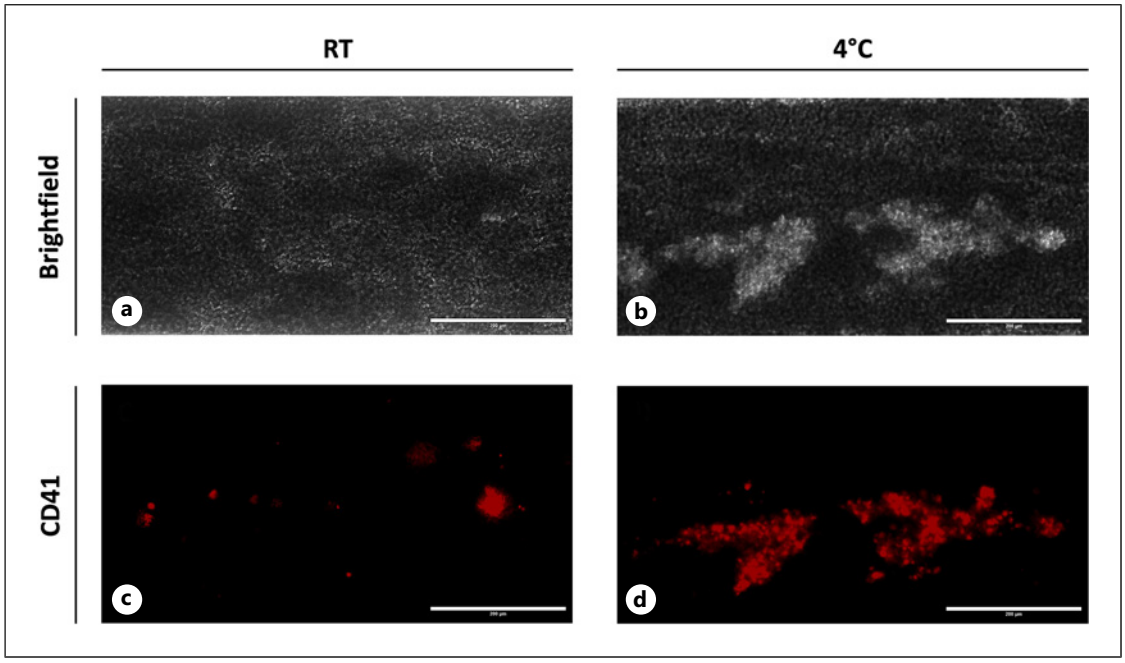

Fig. 2. Ex vivo thrombus formation of room temperature-versus 4°C-stored platelet concentrates. Representative brightfield (upper panel) and immunofluorescence pictures (lower panel) of ex vivo thrombi formed on collagen-coated channels. Spike-in platelet concentrates, stored at room temperature (RT, **a** and **c**) or at 4°C (**b** and **d**) were perfused through microfluidic channels, upon TRAP stimulation, for 5 min. Prior to perfusion samples were stained with CD41 antibody. Red signal: CD41. Scale bar, 200 μm.

thrombosis in patients receiving these products are legitimate. However, it has been shown that apheresis CSPs are still responsive to endothelial inhibitors, like nitric oxide and prostacyclin [35]. These results suggest that refrigerated platelets maintain their ability of hemostatic control at the damage site, without triggering thrombosis, despite their superior activation levels.

Nevertheless, it is important to indicate that methods concerning platelet collection and preparation (apheresis or pooled PCs) as well as storage conditions (composition of the additive solution and storage with or without agitation) varied slightly between the studies mentioned and might have an impact on the results of the study [36]. For example, Reddoch et al. [35] compared metabolic parameters as well as platelet functionality of refrigerated apheresis-derived PCs collected with two different platelet isolation systems which achieve platelet isolation by different centrifugation procedures. Moreover, differently manufactured platelet additive solutions (PASs) were used although the final PAS/plasma-composition was similar (65% PAS/35% plasma) between the two groups. A difference between both PCs regarding in vitro hemostatic functions, adhesion under flow, clot strength, and platelet count was observed over the storage time of 15 days [36]. Additionally, the relevance of the composition of PAS, especially the residual plasma concentration, for CSPs functionality was investigated by our group, and we observed a decrease of in vitro platelet functions when the residual plasma concentration was as low as 20% [16].

Adhesion and Aggregation Abilities of CSPs

We and other groups have shown that CSPs better preserved their ability to adhere to thrombogenic surfaces, such as collagen, more than RT-stored platelets [16, 37]. This might be supported by the enhanced free cytosolic calcium level observed during cold storage [38]. Increased cytosolic calcium causes actin filament fragmentation and cytoskeleton rearrangement which in turn leads to a morphological change from a normal discoidal shape (resting platelets) to a spherical shape (early stage of platelet activation and adhesion) [38, 39]. Intracellular calcium level is not the only reason for the increased CSP functions. Recently, Koessler et al. [15] and his group reported enhanced responsiveness of CSPs, in terms of activation, adhesion, and aggregation due to stabilization of the activity of the purinergic platelet receptor P2Y12 (activated by ADP binding) and concomitant attenuation of inhibitory intracellular pathways [15, 37]. They reported that improved response to ADP, after 6 days of cold storage, was associated with reduced phosphorylation of the VASP [15], which is known to negatively regulate platelets functions [40, 41]. In a subsequent study, they analyzed the impact of short-term refrigeration (1–2 h) on PCs in order to address the

Table 1. Different sources of platelet concentrates and their characteristics

Product's characteristics	Sources of platelets		
	single blood donation*	pooled buffy coats**	single donor platelet apheresis**
Platelet count	~0.8×10^{11}	~3×10^{11}	~3×10^{11}
Platelet concentration/mL	~5×10^{9}	~$1–1.6 \times 10^{9}$	~$1–1.5 \times 10^{9}$
Product final volume	~50–70 mL	~240–300 mL	~220–230 mL
Content of Leukocytes	~$2–2.1 \times 10^{5}$	~$2–2.8 \times 10^{5}$	~$2.4–2.8 \times 10^{5}$

*References 44–46. ** Reference 47.

Table 2. Chemical composition of different additive solutions

Chemical components	Additive solutions			
	PAS-II*	PAS-III**	PAS-E***	PAS-F***
Acetate	30 mM	30 mM	33 mM	27 mM
Citrate	10 mM	10 mM	11 mM	–
NaCl	115 mM	77 mM	–	–
Phosphate	–	26 mM	28 mM	0.5 mM
Impact on platelet metabolism and functionality	Increase in glucose consumption and lactate levels, increase in spontaneous CD62p exposure	No macroscopic aggregates, decrease in glucose, increase in spontaneous CD62p exposure	Higher pH compared to PAS-F, no visible aggregates during storage	Increased aggregate formation during storage and upon ADP stimulation, faster clot formation, higher CD62p exposure compared to PAS-E

*References 48, 51, 52. **Reference 49. ***Reference 50.

time-dependent onset of cold-induced effects that contribute to superior platelet reactivity. In accordance with the previous findings, enhanced ADP- and collagen-induced aggregation as well as increased adhesion to collagen-coated surfaces under flow conditions were accompanied with decreased VASP phosphorylation, already after 1 h of cold storage [37]. Interestingly, in a more recent study, we detected a drastic reduction of CSPs functions, like aggregation and clot stability, upon incubation of the platelets with an activator of the protein kinase A (PKA), which directly phosphorylates VASP, compared to untreated CSPs [42]. Therefore, even our study confirms that the attenuation of inhibitory pathways observed during cold storage might be a key mechanism for the better responsiveness of CSPs.

The Impact of Cold Storage on Clot and Thrombus Formation

In order to reach an efficient hemostatic response, after activation and adhesion, platelets must aggregate ending with clot formation. It has been observed that CSPs better maintained their aggregation ability, upon exposure to several inductors, like collagen or ADP, than RT-stored platelets [16]. Interestingly, the superior aggregation capability is followed by the formation of clots that show higher stability, compared to PCs stored at RT. In fact, as reported by Nair and colleagues [43], which performed scanning electron microscopy analyses, CSPs are able to form denser clots with thinner fibers and more crosslinks, suggesting a rationale for the better cell functionality upon cold storage.

Nevertheless, recent in vitro data reported contradictory results on the superior hemostatic functions of CSPs [44]. In fact, on one hand, it has been demonstrated that the size of thrombi generated under shear stress as well as on a static single-cell assay was significantly larger with refrigerated platelets compared to RT [44]. However, this was not associated with increased contractile forces. Furthermore, reduced glycoprotein VI (GPVI) levels in mouse and human platelets were detected during cold storage. In addition, transfusion experiments using human volunteers under dual antiplatelet therapy showed significantly reduced response to collagen after transfusion of CSP compared to RT, corroborating the in vitro data of decreased GPVI expression. Nevertheless, it is important to keep in mind that the in vitro aggregation assays in response to collagen were performed with fresh washed platelets and that only 8 participants were

enrolled in the human study [44]. Therefore, additional investigations are needed to clarify these points. Moreover, our working group is currently investigating the impact of cold storage on the ex vivo thrombus formation under shear stress, using a multichannel system which resembles the physiological blood flow. Based on our data, as reported in Figure 2, enhanced thrombus formation, defined as surface area covered by thrombi, was observed upon cold storage in comparison to PCs stored at RT. Indeed, these preliminary findings support the superior hemostatic functions of CSPs.

In summary, despite all efforts made in the last decades, it is still difficult to draw a final conclusion on the impact of cold storage on platelet functions since the information obtained from different studies are limited to the specific platelet preparation procedure, product composition (like plasma volume or additive solutions) and storage condition used for performing the study. In Tables 1 and 2, we reported the most common sources of platelets as well as additive solutions used for PCs, respectively [44–52]. In this context, a global standardization of all steps involved in both processes, production and storage, might be extremely helpful to clarify the discrepancies observed until now and take the final decision on the use of CSPs for clinical application.

Conclusion

In modern medicine, transfusion of PCs is a standard therapy used for both prophylactic and therapeutic purposes to prevent or stop bleeding. Indeed, quality and functionality of the transfused platelets are crucial aspects that have to be considered to reach the best patient outcome. Unfortunately, the current storage condition shows severe limitations in terms of high risk of bacterial infection posttransfusion and reduced cell functions. In this context, cold storage has been intensively investigated as alternative storage condition and available data on the efficacy of CSPs in patients with acute bleeding is promising. However, despite numerous studies have reported superior hemostatic functionality of CSPs in vitro, the debate is still open due to the lack of standardization of the processes, like production and storage, which does not allow a clear comparison between the different studies. Therefore, further investigations and clinical studies are necessary to verify the feasibility of this alternative storage temperature which might begin a new chapter in the transfusion medicine.

Conflict of Interest Statement

T.B. is an employer of the Blood Donation Service of the German Red Cross. T.B. has received research funding from CoaChrom Diagnostica GmbH, DFG, Robert Bosch GmbH, Stiftung Transfusionsmedizin und Immunhämatologie e.V.: Ergomed, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium fuer Wissenschaft, Forschung und Kunst Baden-Wuerttemberg, has received lecture honoraria from Aspen Germany GmbH, Bayer Vital GmbH, Bristol-Myers Squibb GmbH & Co., Doctrina Med AG, Meet The Experts Academy UG, Schoechl medical education GmbH, Mattsee, Stago GmbH, Mitsubishi Tanabe Pharma GmbH, Novo Nordisk Pharma GmbH, has provided consulting services to: Terumo has provided expert witness testimony relating to heparin-induced thrombocytopenia and non-heparin-induced thrombocytopenic and coagulopathic disorders. All of these are outside the current work. T.B. together with DRK Blutspendedienst Baden-Wuerttemberg-Hessen has a pending patent application on the use of apoptosis inhibitors for cold storage of blood platelets. Other authors declare no competing financial interests.

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Author Contributions

Johanna Kirschall, Günalp Uzun, Tamam Bakchoul, and Irene Marini wrote and approved the manuscript. Data presented in this manuscript are a part of the doctoral thesis of Johanna Kirschall.

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