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Short communication

CLEARANCE OF TREHALOSE LYOPHILIZED PLATELETS IN MICE

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The utilization of stored platelet transfusion has emerged as an effective approach in the management of thrombocytopenia. However, the limited availability of fresh platelets in veterinary medicine due to the challenging storage requirements poses a significant constraint. Lyophilized platelets offer extended shelf-life and convenient storage options, enhancing their accessibility for thrombocytopenic patients. However, only a limited number of studies have examined the clearance and survival rate of transfused lyophilized platelets, particularly regarding the lyophilization technique utilizing trehalose as a platelet stabilization agent. The objective of this study was to assess the recovery and survival rate of trehalose lyophilized platelets within the circulatory system. To investigate these parameters, CMFDA-labeled lyophilized platelets were administered to mice, and their recovery and survival rates were analyzed. Flow cytometric analysis revealed the rapid clearance of lyophilized platelets from the systemic circulation. The immediate post-infusion percent recovery of labeled platelet particles was 42.7 ± 8.15 %. The average survival rates at post-infusion time points at 15, 30, 45, and 60 minutes were 28.2 ± 4.31, 14.5 ± 3.56, 5.1 ± 2.02, and 0.82 ± 0.57, respectively. The calculated mean half-life was 8.39 ± 0.44 minutes. The most pronounced decrease in labeled lyophilized platelet count occurred during the 30-minute timeframe immediately following infusion. Subsequently, over 99% of lyophilized platelets were eliminated after 60 minutes post-infusion. These findings indicate that higher dosages and more frequent administration of trehalose lyophilized platelets might be necessary to achieve a therapeutic effect comparable to that of fresh platelets.

Keywords: clearance, lyophilization, mouse, platelet, survival rate, trehalose

INTRODUCTION

Platelet transfusion is widely used to prevent and treat bleeding in thrombocytopenic patients. However, the transfusion of platelets in veterinary medicine is predominantly restricted to platelets in freshly obtained whole blood, primarily due to challenges associated with collecting and storing platelets [1]. Fresh platelets have a limited

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storage duration of 5 days before transfusion, necessitating continuous agitation at a temperature range of 20-24 °C for optimal preservation. Various strategies have been developed to enhance the clinical viability of platelets and overcome practical limitations such as inadequate blood donors, low production efficiency, and limited shelf life [2-5]. Recently introduced lyophilized platelet products have demonstrated several advantages in terms of prolonged shelf life and preservation of platelet functionality [5,6]. In addition, the storage of lyophilized platelets in a dehydrated state renders them less prone to bacterial contamination throughout the storage phase.

Several formulations have been devised to protect platelets from potential damage during lyophilization. In the past, the most common formulations included the fixation of platelets using paraformaldehyde, followed by lyophilization in a serum albumin solution [2,7]. However, due to their potential toxicity and carcinogenicity, other formulations have begun to be more commonly used. Utilizing the natural cryoprotectant trehalose, platelets can be successfully lyophilized without experiencing significant structural and functional alterations [8]. Trehalose agents stabilize platelet membranes and inhibit the formation of detrimental ice crystals by replacing water content within the platelets [9]. In contrast to fresh platelets, which are viable for 5 days, lyophilized platelets can be stored for up to 2 years [10].

In the case of cryopreserved or lyophilized platelets, a considerably higher quantity of transfused platelets is necessary to achieve a comparable platelet increment compared to fresh platelets, primarily due to the loss of numerous platelets during freeze-thaw and lyophilization procedures [8,11-13]. Furthermore, post-transfusion, cryopreserved or lyophilized platelets experience more rapid clearance from the systemic circulation than fresh platelets [7,14].

A significant proportion of the studies associated with platelet lyophilization were conducted with platelets prepared with paraformaldehyde. In contrast, only a limited number of studies have been conducted on using trehalose for platelet lyophilization. No studies assessed the in vivo survival rate of trehalose lyophilized platelets within the circulation, a crucial aspect for practical application. The present study aimed to evaluate the time-dependent in vivo clearance of trehalose lyophilized platelets.

MATERIALS AND METHODS

Animals

Female specific-pathogen-free C57BL/6J mice, weighing 35-38 g, were used with approval from the Institutional Animal Care and Use Committee at Konkuk University (Approval number KU21059). Eighty mice were housed in groups of five animals in filter cages and fed standard rodent chow and tap water.

Preparation for Lyophilized Platelets

Thirty mice were anesthetized with intramuscular injectinos of zoletil (20 mg/kg) and xylazin (10 mg/kg) for all blood collections. The abdomen was cut open and the maximal blood volume (1.5 ml) was drawn from the Vena cava through a 25-gauge needle into a 3 ml syringe containing 150 μl of 3.2% w/v sodium citrate. The final dilution ratio between blood and anticoagulant was 9:1 (v/v). All mice were euthanized by cervical dislocation after the blood collection. Lyophilized platelets were prepared as previously described with centrifugal cell separation steps [15]. Briefly, the thirty fresh anticoagulated blood samples were pooled in pools of 5 individual samples and centrifuged at 750×g for 3 minutes to harvest platelet-rich plasma (PRP). The PRPs were pooled together and recentrifuged at 1300×g for 10 minutes to produce a platelet pellet that was suspended in Tyrode's buffer (9.5 mM HEPES, 100 mM NaCl, 4.8 mM KCl, 12 mM $NaHCO_3$, 10 μg/μl prostaglandin E1, and 1% ethanol) containing 50 mM trehalose, pH 6.8, to a final platelet concentration of approximately 5×10^8 platelets/ml. The platelet solution was incubated at 37 °C for 4 hours and bovine serum albumin was added to a final concentration of 5%. The platelet solution was frozen and subsequently lyophilized. Primary drying was performed at -40 °C under 20 mTorr for 16 hours, and during secondary drying, the shelf temperature was increased up to 22 °C at a rate of 0.2 °C/minute. The samples were kept at 22 °C for 16 hours. Throughout the whole lyophilization process, the vacuum was maintained at 4 mTorr.

In Vivo Platelet Survival

Lyophilized platelets were rehydrated in distilled water and labeled with 2.5 μM Cell Tracker CMFDA for 30 minutes (22 °C) in the dark. Fifty female C57BL/6j mice were infused with the CMFDA-labeled lyophilized platelets ($3\text{-}3.5\times10^8$) in 0.2 ml distilled water via the lateral tail vein. 100 μl of whole-blood samples of all fifty mice were collected from the retroorbital venous plexus immediately (< 2 minutes) after infusion of labeled platelets. Platelet counts were measured using an automated hematology analyzer. For survival determination, additional blood samples (50-100 μl) were collected from the contralateral retroorbital venous plexus of ten mice at each time point, including 15, 30, 45, and 60 minutes after labeled platelets infusion. The percentage of CMFDA-labeled platelets was determined using flow cytometry. The percent of recovery (R) was expressed as previously described [16]. Briefly, %R was calculated by the formula:

$$\%R = \text{Total number of circulating labeled platelets} \div \text{Total number of transfused labeled platelets}$$

A total of 50,000 platelets was analyzed in each sample, and the maximum number of labeled platelets measured at first (< 2 minutes) was set as 100%. The sequential survival rates were expressed as a percent of the maximum.

Statistical analysis

The platelet survival rate test values were analyzed to evaluate the time-dependent changes in the level of circulating CMFDA-labeled platelets in mice. The exponential regression was fitted to the data, and the circulating half-life ($T_{1/2}$) was calculated from the survival curve. All the experimental values are presented as the mean and standard deviation.

RESULTS AND DISCUSSION

Survival of mice lyophilized platelets in the circulation was determined by examining the circulatory clearance kinetics. The mice were infused with the CMFDA-labeled lyophilized platelets equivalent to approximately 15% of the recipient platelet mass. Blood samples were collected at each designated time point over one hour and subsequently analyzed using flow cytometry to quantify the number of labeled platelets out of a total pool of 50,000 platelets (Figure 1). The mean platelet count of the recipient mice was $1.219 \pm 0.39 \times 10^9$/ml. The mean percent recovery of labeled platelet in the circulation immediately after infusion was 42.7 ± 8.15 %. The average survival rates at post-infusion time points at 15, 30, 45, and 60 minutes were 28.2 ± 4.31, 14.5 ± 3.56, 5.1 ± 2.02, and 0.82 ± 0.57, respectively (Figure 2). The mean half-life was 8.39 ± 0.44 minutes. The most significant decline in labeled platelet count was observed within the 30-minute period following infusion.

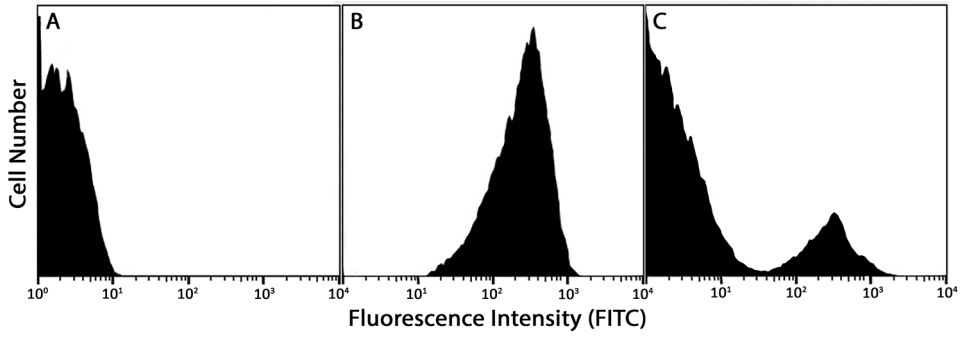

Figure 1. Relative fluorescence intensity of CMFDA-unlabeled **(A)** and labeled **(B)** lyophilized platelets. **(C)** A blood sample obtained immediately after the infusion (< 2 minutes) reveals a bimodal distribution.

In the current study, the recovery and survival rates of trehalose lyophilized platelets showed a significant decrease following infusion, in accordance with previous literature demonstrating the rapid clearance of lyophilized platelets from systemic circulation compared to fresh platelets [14,17,18]. Immediately after injection, approximately 57% of the infused lyophilized platelets were lost, significantly lower than the mean maximum in vivo platelet recovery rate (80.3%) for fresh platelet infusion [11]. The half-life was 8.39 minutes, indicating that the lyophilized platelets were cleared from

the recipient's circulation at an exponential rate. After 60 minutes post-infusion, more than 99 % of lyophilized platelets were removed. These results might be related to structural impairment and changes caused by the lyophilization process, rendering the platelet susceptible to physical damage or facilitating recognition and clearance by the recipient's immune system [18]. The clearance of infused platelets from the circulation involves multiple mechanisms, with desialylation identified as a major contributing factor. To address the issue of rapid clearance, various methods can be employed, such as the administration of immunomodulatory agents (including intravenous immunoglobulin G, corticosteroids, vincristine, and anti-RhD therapy), neuraminidase inhibitors (DANA, oseltamivir phosphate), ASGPRs competitor (asiolofetuin), and Fcγ fragments [18,19]. These agents could hold the potential to enhance the effectiveness of lyophilized platelet transfusion.

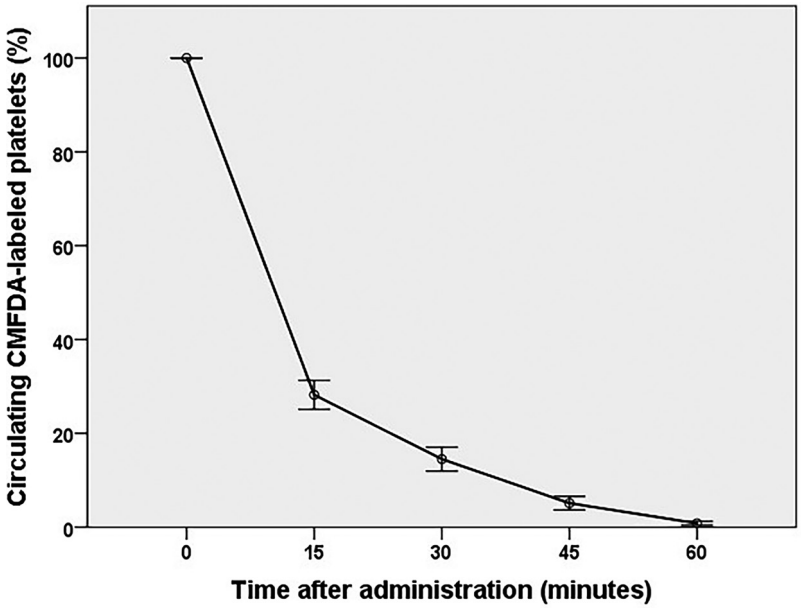

Figure 2. Survival kinetics of trehalose lyophilized platelets in the circulation of mice. The percentage of labeled platelets in each blood sample was determined by analyzing 50,000 platelets, with the number of labeled platelets measured at first (< 2 minutes) as the baseline reference point of 100%. The subsequent survival rates are expressed as a percentage relative to the baseline.

The major limitations of this study are the lack of comprehensive elucidation of rapid clearance mechanisms and the absence of an evaluation of the lyophilized platelet's hemostatic function. The observed low recovery and survival rates can be attributed to several factors, including potential destruction during needle injection, rapid consumption, and immune reactions, such as macrophage phagocytosis. To effectively translate the findings of this study into clinical practice within hospitals, it is essential to establish a definitive correlation between the survival kinetics and hemostatic

properties of lyophilized platelets. In addition, in the case of thrombocytopenic patients, the coagulability of blood is commonly impaired, changing the platelet's survival kinetics and therapeutic effects. Therefore, the recipient's hemodynamic and hemostatic state should also be considered when assessing the correlation. In addition, this study has limitations in that blood samples were collected from different mice at each time point, instead of serially collecting blood samples from the same mice over multiple time points. Serial blood sampling from the same animal is the most reliable way to monitor the temporal behavior of infused lyophilized platelets. However, repetitive blood sampling from individual mice within a short time frame could induce stressful stimuli, which would affect hemodynamics and hemostasis. To mitigate this potential confounding factor, the current study limited blood sampling to two time points per mouse. The first group of ten mice provided a single whole blood sample immediately after infusion, while the remaining forty mice underwent two blood collections each, one immediately post-infusion and the other at predetermined time intervals thereafter. Further study is warranted to evaluate the time-dependent clearance of lyophilized platelets in individual mice without causing the detrimental effect of repetitive blood sampling.subjecting them to the adverse effects of repeated sampling.

In conclusion, comparable to the other platelet lyophilization formulations involving formaldehyde or paraformaldehyde, the use of trehalose in platelet lyophilization also resulted in rapid clearance from systemic circulation. These findings indicate that, similar to other platelet stabilizing agents, trehalose is not entirely effective in completely preventing the structural or functional impairment caused by the lyophilization process. Therefore, given the lower in vivo survival rate and shorter half-life, the higher dosages and more frequent administration of trehalose lyophilized platelets might be necessary to achieve a comparable therapeutic effect to fresh platelets. Further studies are needed to assess the therapeutic effects of trehalose lyophilized platelets. The authors expect that this study lays the groundwork for advanced comparative studies and clinical applications.

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Authors' contributions

MYK carried out the *in vivo* lyophilized platelet survival studies and drafted the manuscript. HJH participated in its design and coordination and helped to draft the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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KLIRENS MIŠIJIH TROMBOCITA LIOFILIZOVANIH U TREHALOZI

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Korišćenje transfuzije uskladištenih trombocita se pokazalo kao efikasan pristup u lečenju trombocitopenije. Međutim, ograničena dostupnost svežih trombocita u veterinarskoj medicini zbog izazovnih zahteva za skladištenje predstavlja značajno ograničenje. Liofilizovani trombociti nude produženi vek trajanja i pogodne opcije skladištenja, poboljšavajući njihovu dostupnost za pacijente sa trombocitopenijom. Međutim, samo ograničen broj studija je ispitao klirens i stopu preživljavanja liofilizovanih trombocita nakon transfuzije, posebno u pogledu tehnike liofilizacije koja koristi trehalozu kao sredstvo za stabilizaciju trombocita. Cilj studije je bio da se proceni u cirkulaciji stopa oporavka i preživljavanja trombocita liofilizovanih trehalozom. Da bi se istražili ovi parametri, miševima su davani liofilizovani trombociti obeleženi CMFDA i analizirane su njihove stope oporavka i preživljavanja. Protočna citometrijska analiza je otkrila brzo uklanjanje liofilizovanih trombocita iz sistemske cirkulacije. Procenat oporavka obeleženih trombocita neposredno posle infuzije bio je 42,7 ± 8,15 %. Prosečne stope preživljavanja u vremenskim tačkama nakon infuzije na 15, 30, 45 i 60 minuta bile su 28,2 ± 4,31, 14,5 ± 3,56, 5,1 ± 2,02 i 0,82 ± 0,57. Izračunati srednji poluživot bio je 8,39 ± 0,44 minuta. Najizraženije smanjenje broja obeleženih liofilizovanih trombocita dogodilo se tokom 30-minutnog vremenskog okvira neposredno nakon infuzije. Nakon toga, preko 99% liofilizovanih trombocita je eliminisano 60 minuta nakon infuzije. Ovi nalazi ukazuju na to da bi veće doze i češća primena liofilizovanih trombocita mogli biti neophodni da bi se postigao terapeutski efekat uporediv sa onim kod svežih trombocita.