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Enhancing Platelet Preservation through Freeze-Drying and Sterilization: An Approach for the Improvement of Hemostatic Treatment and Platelet Concentrate Supply

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Abstract

Background: The limited shelf life of platelets has led to an increasing demand for longer-lasting products. This study aimed to develop a lyophilization protocol to preserve platelets by using trehalose, a desiccation-resistant sugar, and comparing its effectiveness to sucrose, a standard sugar for protein lyophilization.

Materials and Methods: In this interventional study, the platelets were loaded with trehalose (30, 60, or 100 mM) and 2% sucrose and then freeze-dried. Evaluations were performed of platelet count, aggregation responses to the agonists thrombin (1 U/ml), collagen (2 µg/ml), adenosine diphosphate (ADP) (20 µM), and the expression of the activation surface marker CD62P. Gamma radiation (30 and 40 kGy) was evaluated for pathogen inactivation in lyophilized products through measuring the reduction factors for viral titers (Herpes Simplex Virus-1 (HSV-1) and Poliovirus) and bacterial titers (S. epidermidis and E. coli). The analyses were conducted using SPSS v23.0.1 and GraphPad Prism v10.

Results: The platelet count in the 60 mM trehalose group showed no significant change after lyophilization (mean difference: 78.33; P = 0.31). The flow-cytometry analysis revealed a significant increase of CD62P in the control and sucrose-treated groups (P < 0.01 for all the groups), while trehalose significantly preserved the platelet function. This was demonstrated by lower CD62P (7.88%, P = 0.000) and higher thrombin-induced aggregation (53.46%, P = 0.01) and ADP-induced aggregation (29.8%, P = 0.001) compared to the other groups. Gamma radiation achieved a 5.2-log reduction at 30 kGy and a 6.2-log reduction at 40 kGy for Poliovirus, along with a consistent 7-log reduction for HSV-1 at both. Additionally, titers of S. epidermidis and E. coli were reduced by more than 7 logs, rendering them undetectable.

Conclusion: Lyophilized platelets stabilized with trehalose and sterilized with gamma radiation represent a promising approach for dealing with the current limitations in platelet storage and availability.

Keywords: Freeze-drying, Gamma rays, Platelet, Sucrose, Trehalose

Introduction

Platelet transfusion is a well-established medical procedure widely used in healthcare settings to prevent or treat bleeding. However, platelet storage is constrained by the risk of microbial growth and platelet storage lesions, necessitating their disposal after five days (or three days in Iran) to comply with federal regulations. This limited storage duration is primarily due to platelet activation at temperatures below 20°C (1). Uncontrolled bleeding resulting from traumatic injuries remains a significant cause of mortality. The current restrictions on platelet shelf life, which requires frequent donations, and limited availability, particularly in remote areas or emergencies, have driven the demand for longer-lasting hemostatic agents (2). Efforts have focused on extending platelet shelf life by optimizing storage conditions and integrating cold storage solutions with platelet additives, which have shown promising results (2). Alternative preservation strategies include hypothermic storage, cryopreservation, the

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use of platelet activation inhibitors, pathogen reduction technologies, and enhanced oxygen-permeable storage bags, all of which exhibit varying levels of effectiveness (3-6). One potential solution involves developing novel methods of lyophilizing platelets to create a readily applicable hemostatic product that requires minimal preparation in trauma settings where conventional treatment options are unavailable. Lyophilization has gained attention in transfusion medicine for its potential to address challenges related to storage, availability, and safety. However, lyophilizing mammalian cells remains a significant challenge despite ongoing research (7). The freeze-drying process compromises both platelet morphology and function. To mitigate the corresponding effects, recent studies have investigated stabilizing techniques designed to preserve the platelet structure and functionality during the lyophilization process. These methods aim to minimize the detrimental impact of freeze-drying and maintain platelet integrity for therapeutic use (8). Initial attempts to lyophilize paraformaldehyde-fixed platelets yielded low recovery rates (7). Subsequent studies reported successful freeze-drying of aldehyde-fixed platelets, which restored hemostasis in thrombocytopenic mice. However, this method requires further optimization and evaluation due to potential complications, such as increased antigenicity and reduced sensitivity to agonists in vitro (9, 10). A promising development is the thrombosome (TS) product, developed by Cellphire Inc., which employs trehalose as a stabilizer during lyophilization (11). Each cryoprotectant offers distinct advantages and challenges, influencing the choice of the most suitable agent for the freeze-drying process. Trehalose, a disaccharide found in organisms, endures extreme dehydration and is particularly effective due to its ability to replace the water shell surrounding macromolecules, thereby preventing damage during the drying process. Its high glass transition temperature facilitates the formation of stable glasses at room temperature, preserving cells in their dried state (12). Similarly, sucrose has been extensively used to preserve biological products due to its strong stabilizing properties (12, 13). In addition to effective cryoprotectants, ensuring the safety and extended preservation of platelets also requires pathogen reduction techniques (14). The existing chemical pathogen reduction techniques are effective against lipid-enveloped viruses but have variable efficacy against non-lipid-enveloped viruses and different bacterial species. Furthermore, these techniques are associated with potential long-term effects from residual inactivating compounds, their degradation products, or their interactions with proteins (14). Gamma irradiation has emerged as an alternative pathogen inactivation method that avoids chemical additives and eliminates a broad spectrum of pathogens while maintaining the structural and functional integrity of biological products. It achieves pathogen neutralization through two primary mechanisms including the direct disruption of covalent bonds and the indirect generation of free radicals and reactive oxygen species through interactions with water molecules and oxygen (15). Optimizing the irradiation dose is essential to achieving an effective balance between pathogen inactivation and the preservation of the therapeutic efficacy of lyophilized platelets. The goal of this study is to develop and optimize a comprehensive method of producing lyophilized platelets, incorporate effective cryoprotectants, and evaluate the efficacy of gamma irradiation for inactivating model viruses and bacteria. The study also addresses the current limitations in platelet storage and availability. By evaluating the impact of

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lyophilization on platelet function and exploring the application of gamma irradiation for pathogen reduction, the present study aims to establish a safe, functional, and readily deployable hemostatic product.

Materials and Methods

Platelet concentrate selection

This interventional study adheres to all the relevant biological guidelines concerning platelet bags obtained via the Platelet-Rich Plasma (PRP) method, following the Standard Operating Procedures (SOPs) of the Iranian Blood Transfusion Organization. The quality of the platelet products was evaluated by measuring the platelet volume and count. Only the bags with a platelet count exceeding one million per microliter, normal platelet volume, and aggregation levels above 70% for all the tested agonists were included in the study. The study protocol was approved by the Research Ethics Committee of the Higher Institute of Research and Medical Education of Blood Transfusion (IR.TMI.REC.1401.009). Informed consent was obtained from all the blood donors.

Preparation and washing of platelets

To evaluate suitable lyophilization storage buffers, the PRP samples were divided into four groups including control, 60 mM Trehalose, 2% Sucrose, and a combination of Trehalose and Sucrose. Initially, PRP was washed with a washing buffer containing 100 mM NaCl, 10 mM KCl, 10 mM imidazole, 10 mM EGTA, and 12 mM $NaHCO_3$ (pH 6.8). The samples were thoroughly mixed with the buffer and centrifuged at 500×g for 5 minutes. The supernatant was then removed and replaced with the lyophilization buffer. The control group consisted of an incubation buffer (100 mM NaCl, 10 mM KCl, 10 mM imidazole, 12 mM $NaHCO_3$, and 3% human serum albumin, pH 6.8) without additives, while the test groups included 60 mM trehalose (sigma-aldrich), 2% sucrose (sigma-aldrich), or a combination of both in the incubation buffer. The tubes were incubated at 37°C for 2 hours, followed by centrifugation to remove the incubation buffer. Lyophilization buffers were added accordingly, and platelet counts were adjusted to exceed 600,000/µl in all the groups.

Lyophilization process

The platelet samples were frozen at -80°C for 5 hours before being transferred to a laboratory-scale freeze dryer. The condenser temperature was maintained at -45°C. The vacuum pressure reached approximately 20 mTorr within an hour. During the drying process, the sample temperature rose to ambient levels in an uncontrolled manner. The samples remained in the lyophilizer for at least 24 hours at room temperature. The lyophilized platelets were reconstituted to their original volume using distilled water, gently rotated for 30 minutes to ensure proper hydration, and assessed within an hour.

Measurement of platelet parameters

The platelet count and the mean platelet volume were measured using a hematology analyzer (Sysmex, K1000) before and after lyophilization.

Evaluation of platelet activation

The lyophilized platelets were incubated with mouse anti-human CD62P antibodies at 20°C for 30 minutes. Flow cytometry was done to identify the platelets based on their forward and side scatter properties, and the percentage of the activated platelets was determined through assessing CD62P-positive platelets.

Platelet aggregation

Platelet aggregation was assessed after adjusting the platelet concentrations using agonists at varying concentrations: thrombin (1 U/ml), collagen (2 µg/ml), and

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ADP (20 µM) (Hyphen, France). Aggregation was measured using an analyzer (Crono-Log Corporation®) that detected changes in light transmission at 740 nm and 37°C. The results were reported in percentages.

Real-time PCR gene expression

The mRNA (messenger ribonucleic acid) expression levels of *BAK* (*Bcl-2* homologous antagonist/killer), *BAX* (*Bcl-2* associated X protein), and *Caspase-3* genes were analyzed. RNA was extracted using the RNeasy Kit (Qiagen, Germany), and the RNA integrity was confirmed using a NanoDrop spectrophotometer (Thermo Scientific). The RNA samples with an OD (optical density) 260/280 ratio of > 1.8 were reverse-transcribed into cDNA (deoxyribonucleic acid) using a cDNA Synthesis Kit (Thermo Scientific). A quantitative Real-Time Polymerase Chain reaction (qRT-PCR) was performed through SYBR green-based detection on a thermocycler (Rotor-Gene 6000, Qiagen). Each reaction included 1.5 µl of cDNA, 1.2 µl of forward and reverse primers, 7.5 µl of RealQ Plus 2 × Master Mix Green (Ampliqon, Denmark), and 4.8 µl of nuclease-free water, for a total volume of 15 µl. The primer specificity was confirmed using the NCBI BLAST database (Table I). *GAPDH* served as the reference gene. Relative gene expression was also calculated using the 2^-ΔΔCT method (16).

Sterilization and pathogen inactivation

Viruses, including enveloped and non-enveloped RNA and DNA viruses, such as Herpes Simplex Virus-1 (HSV-1) and the Poliovirus vaccine, were obtained from the Virology Laboratory of the Iranian Blood Transfusion Organization. To increase the initial viral titer, the viruses were cultured in Vero host cells. After propagation in the host cells, the viral titer was determined using the $TCID_{50}$ (median tissue culture infectious dose) method based on the Reed and Muench procedure, with serial dilutions (logarithmic base 10) performed in quadruplicate. The $TCID_{50}$ represents the dilution at which 50% of the host cells exhibit CPE (cytopathic effects) when exposed to the virus. Two bacterial species, identified as the most common contaminants in blood transfusion, were selected. They were Staphylococcus epidermidis (ATCC: 25922), a Gram-positive bacterium, and Escherichia coli (ATCC: 25923), a Gram-negative bacterium. To achieve an initial titer of 10^6 CFU/ml, a suspension of each bacterium was prepared in buffer, and the titer was calculated based on OD measurements. The viral and bacterial models were then added to platelet concentrates. The negative controls consisted of pathogen-free samples, while the positive controls contained pathogens without exposure to the PI (pathogen inactivation) method. The samples were lyophilized according to the established protocol and subjected to gamma irradiation at doses of 30 kGy and 40 kGy using cobalt-60 sources. Gamma irradiation was performed at the Atomic Energy Organization of Iran in Tehran. Following the PI process, the secondary viral titers were determined using Vero host cells cultured in 96-well plates. Those titers were calculated using the Reed and Muench formula and reported as $TCID_{50}$. The RF (reduction factor) was calculated with the following equation:

$$R = \log 10A0 - \log 10At$$

Where R represents the reduction factor, $A0$ is the initial viral titer, and At is the secondary viral titer after PI.

To assess bacterial contamination, the samples were spiked with bacterial suspensions so as to achieve an initial titer of 10^5 to 10^6 CFU/ml (Culture Forming Unit). After gamma irradiation, the samples were cultured in specific growth media to evaluate the bacterial survival.

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Statistical analysis

The normality of the data was assessed using the shapiro-wilk test. The results were reported as mean ± standard deviation (SD) or median (range). The group comparisons were performed using Tukey's multiple comparison tests for parametric data and Kruskal-Wallis H tests for non-parametric data. A significance level of $P \leq 0.05$ was considered statistically significant. The analyses were conducted with SPSS v23.0.1 and GraphPad Prism v10.

Results

Changes in the mean platelet volume and count in the studied groups after lyophilization

To evaluate the cellular changes before and after lyophilization, the group treated with 60 mM trehalose showed numerically better outcomes. As illustrated in Figure 1, the platelet count decreased in all the groups after lyophilization. However, the reduction was statistically significant in the sucrose group (mean difference of 233.3, $p = 0.0017$), control group (mean difference of 341.0, $p < 0.0001$), and sucrose-trehalose combination group (mean difference of 245.0, $p = 0.0012$) ($p < 0.05$), whereas the 60 mM trehalose group (mean difference of 78.33, $p = 0.31$) did not exhibit significant changes. Figure 2 shows the changes in the platelet volume. No statistically significant differences were observed in the platelet volume between the treated groups and the control group before and after lyophilization. All the groups had a slight increase in their platelet volume after lyophilization compared to the pre-lyophilization levels, but the mean values for 60 mM trehalose remained unchanged.

CD62P expression in the studied groups after lyophilization

All the cryoprotectant-treated groups (60 mM trehalose: 7.88%, $p = 0.000$, 2% sucrose: 23.79%, $p = 0.000$, and sucrose-trehalose combination: 19.99%, $p = 0.000$) exhibited significantly lower levels of CD62P expression compared to the control group after lyophilization (Figure 3). Among them, the reduction in CD62P expression was most pronounced in the trehalose group (7.88%, $p = 0.000$); there was a statistically significant difference compared to the other groups.

Platelet aggregation after lyophilization in response to agonists

The platelet aggregation assay with a thrombin agonist demonstrated that the platelets lyophilized with 60 mM trehalose had significantly enhanced aggregation compared to the control and the other groups (53.46% in trehalose vs. 14.06% and $p = 0.000$ in the control, 23.16% and $p = 0.003$ in the sucrose-trehalose, and 19.43% and $p = 0.001$ in the sucrose groups). In contrast, the sucrose-treated groups exhibited noticeable inhibitory effects on aggregation compared to trehalose-treated group (Figure 4). Similarly, ADP-induced aggregation was significantly better in the trehalose group (29.8%) compared to the control (3.7%, $p = 0.001$) and the other groups (6.16%, $p = 0.002$ and 4.83%, $p = 0.001$ for sucrose-trehalose and sucrose, respectively). The sucrose-treated groups, either alone ($p = 0.993$) or in combination ($p = 0.935$), had no significant differences from the control. The groups were not significantly different in terms of collagen-induced platelet aggregation, with the aggregation remaining below 10% across all the groups (p-value > 0.05). To confirm these findings, the concentration of trehalose was further optimized and reassessed.

CD62P expression and platelet aggregation at different trehalose concentrations

The statistical analysis revealed significantly lower CD62P expression at 30, 60, and 100 mM trehalose

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concentrations compared to the control. Among them, the 60 mM concentration had the lowest expression (5.56% ± 1.1, p = 0.000) compared to 100 mM (14.21% ± 1.5, p = 0.008) and 30 mM (24.19% ± 0.73, p = 0.000) (Figure 5).

For thrombin-induced aggregation, 60 mM trehalose (41.48% ± 7.7) and 100 mM trehalose (31.08% ± 3.7, p = 0.07) were not significantly different, but both achieved significantly higher aggregation compared to 30 mM trehalose (17.54% ± 1.89, p = 0.001) and the control group (9% ± 2.3, p = 0.000) (Figure 6). ADP-induced aggregation showed consistent results across 30 mM (19.4% ± 2.21), 60 mM (25.23% ± 1.55), and 100 mM (22.56% ± 3.18) concentrations, all significantly higher than the control group (p = 0.000). Collagen-induced aggregation remained unchanged across all the groups and concentrations, with values below 10% (p-value > 0.05).

Expression levels of pro-apoptotic genes (*BAK*, *BAX*, and *Caspase-3)*

The expression of pro-apoptotic genes *BAK*, *BAX*, and *Caspase-3* was at similar levels in the 60 mM trehalose-treated and lyophilized control groups. Both groups exhibited higher gene expression compared to fresh control platelets (p-value > 0.05). However, no statistically significant difference was found between the 60 mM trehalose group and the lyophilized control group (Table II).

Platelet aggregation after one month of storage at room temperature and 4°C

The platelets stored for one month at 4°C retained functional aggregation in response to all the agonists, with no significant changes observed (p > 0.05 for all the agonists). In contrast, the platelets stored at room temperature (RT) declined in ADP-induced aggregation (t = 20.57, df = 2, p = 0.002) over time, while the aggregation responses to collagen remained unchanged. Thrombin-induced aggregation also decreased at RT, though this change was not statistically significant (p > 0.05) (Figure 7).

Pathogen inactivation (PI) in lyophilized platelet products

For viruses, lyophilization alone reduced the titers of Polio and HSV-1 by 2 and 2.6 logs, respectively. Gamma radiation further reduced the viral titers to below the limit of detection (< 1 TCID50/mL) at 30 and 40 kGy doses, achieving reductions of 5-7 logs depending on the virus (Table III). There was a 5.2-log reduction in Poliovirus and a 7-log reduction in HSV-1 at 30 kGy, while there occurred a 6.2-log reduction in Poliovirus and a 7-log reduction in HSV-1 at 40 kGy. For bacterial pathogens, lyophilization alone had no significant impact on bacterial loads. However, gamma radiation effectively reduced the titers of S. epidermidis and E. coli by more than 7 logs, bringing them below the detection limit (Figure 8).

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Table I: The sequence of the primers used

Gene		Sequence	Sequence amplified	Product size (bp)
BAK	F	ATGGTCACCTTACCTCTGCAA	XM_011514780.2	92
	R	TCATAGCGTCGGTTGATGTCG		
BAX	F	CCCGAGAGGTCTTTTTCCGAG	NM_001291431.2	155
	R	CCAGCCCATGATGGTTCTGAT		
Caspase3	F	ATGGAAGCGAATCAATGGA	NM_001354782.2	137
	R	TGTACCAGACCGAGATGTC		

bp: base pair, *BAK*: Bcl-2 homologous antagonist/killer, *BAX*: Bcl-2 associated X protein

Table II: Comparison of the study groups in terms of gene expression profile

ANOVA multiple comparison		N	Mean	p-value
BAK	**lyophilized control**	3	14.08 ± 5.93	0.046
	lyophilized PRP	6	7.85 ± 4.48	
	PRP	3	4.46 ± 2.02	
	Total	12	8.56 ± 5.42	
BAX	**lyophilized control**	3	8.34 ± 3.27	0.111
	lyophilized PRP	6	5.11 ± 2.63	
	PRP	3	3.49 ± 1.25	
	Total	12	5.51 ± 2.96	
Caspase3	**lyophilized control**	3	9.68 ± 5.88	0.904
	lyophilized PRP	6	8.92 ± 4.42	
	PRP	3	7.95 ± 3.93	
	Total	12	8.87 ± 4.29	

BAK: Bcl-2 homologous antagonist/killer, *BAX*: Bcl-2 associated X protein, *PRP*: Platelet Rich Plasma, *ANOVA*: Analysis of Variance

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Table III: The impact of pathogen inactivation method

Pathogen	Polio			HSV-1			E.Coli			S.Epidermidis		
Titration (Log10/mL)	RF	After	Before	RF	After	Before	RF	After	Before	RF	After	Before
Lyophilization	1	6.2	7.2	1.6	7	8.6	0.5	6.5	7	0.3	6.7	**7**
Gamma 30 kGy	5.2	1	6.2	7	<1	7	7	<1	7	7	<1	**7**
Gamma 40 kGy	6.2	<1	6.2	7	<1	7	7	<1	7	7	<1	**7**

HSV-1: Herpes Simplex Virus-1, *RF*: Reduction Factor

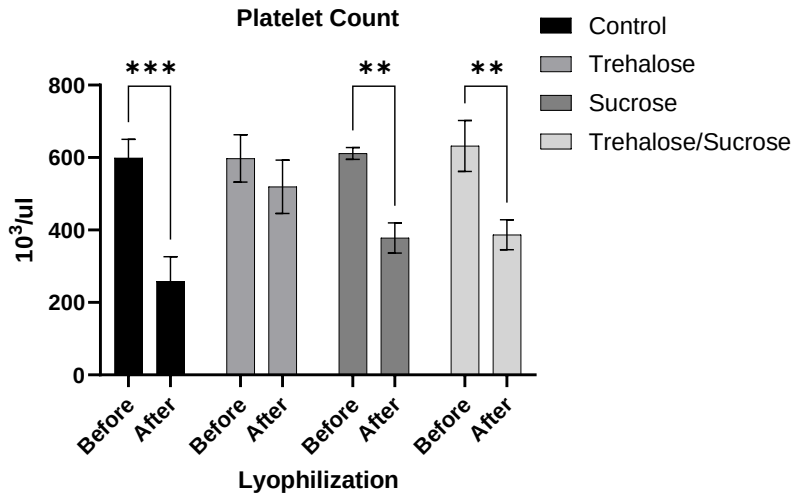

Figure 1. **Platelet count changes after lyophilization:** Statistically significant changes were observed in the sucrose group (mean difference: 233.3, p-value = 0.0017), the control group (mean difference: 341.0, p < 0.0001), and the sucrose-trehalose combination group (mean difference: 245.0, p-value = 0.0012). However, the 60 mM trehalose group showed no significant change (mean difference: 78.33, p-value = 0.31). ** p-value < 0.01, *** p-value < 0.001

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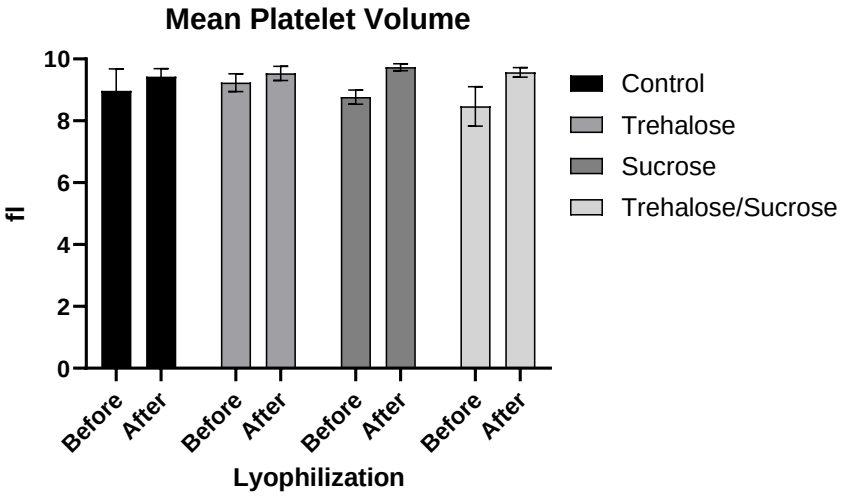

Figure 2. **Platelet volume changes after lyophilization:** The platelet volumes before and after lyophilization were compared using RM two-way ANOVA. No statistically significant differences were observed between the treated groups and the control group.

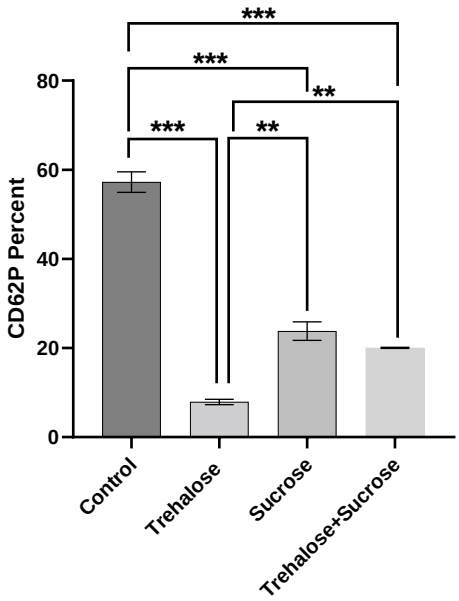

Figure 3. **CD62P expression levels:** Surface marker CD62P expression levels were compared using ANOVA. All the cryoprotectant-treated groups showed significantly lower CD62P expression levels compared to the control group after lyophilization ($p = 0.000$ for all the groups). Trehalose significantly showed lower CD62P expression compared to all the treated groups (7.88%, $p = 0.000$). ** $p < 0.01$, *** $p < 0.001$

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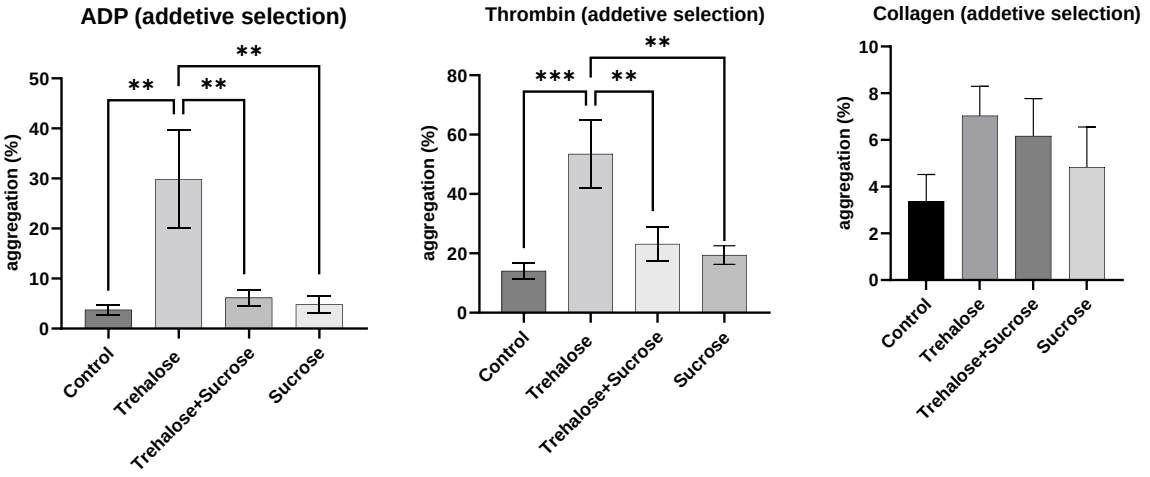

Figure 4. **Platelet aggregation by agonists:** The levels of platelet aggregation in response to thrombin, ADP, and collagen agonists were compared among the cryoprotectant-treated groups and the control group. The 60 mM trehalose group demonstrated superior ADP (trehalose group (29.8%) compared to the control (3.7%, p-value = 0.001) and sucrose-trehalose (6.16%, p = 0.002) and sucrose (4.83%, p = 0.001) groups and thrombin (53.46% in trehalose vs. 14.06%, p = 0.000 in the control, 23.16%, p = 0.003 in the sucrose-trehalose, and 19.43%, p = 0.001 in the sucrose groups) aggregation results compared to all the studied groups. ** p < 0.01, *** p < 0.001

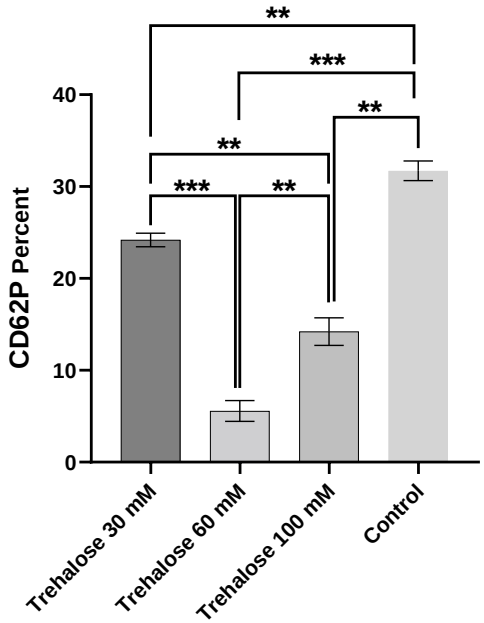

Figure 5. **CD62P expression at different trehalose concentrations:** CD62P expression levels were evaluated at various trehalose concentrations after lyophilization. Significantly lower CD62P expression was observed at 60 mM (5.56% ± 1.1, p-value = 0.000) and 100 mM trehalose (14.21% ± 1.5, p = 0.008) compared to the 30 mM trehalose (24.19% ± 0.73, p = 0.000) and control (31.72% ± 0.61, p = 0.000) groups. ** P-value < 0.01, *** P-value < 0.001

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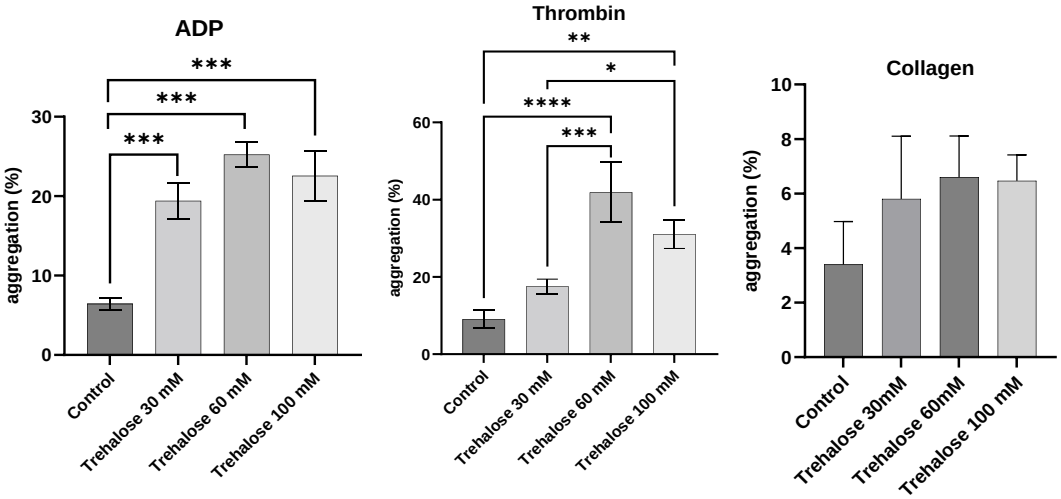

Figure 6. **Platelet aggregation at different trehalose concentrations:** At a concentration of 60 mM (41.48% ± 7.7), trehalose showed no significant difference in aggregation compared to 100 mM (31.08% ± 3.7, p = 0.07). However, both concentrations resulted in significantly higher aggregation compared to 30 mM (17.54% ± 1.89, p = 0.001) and the control group (9% ± 2.3, p = 0.000). ADP-induced aggregation showed no significant results at the trehalose concentrations of 30 mM (19.4% ± 2.21), 60 mM (25.23% ± 1.55), and 100 mM (22.56% ± 3.18); they were all significantly higher than those observed in the control group (p = 0.000).
* P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001

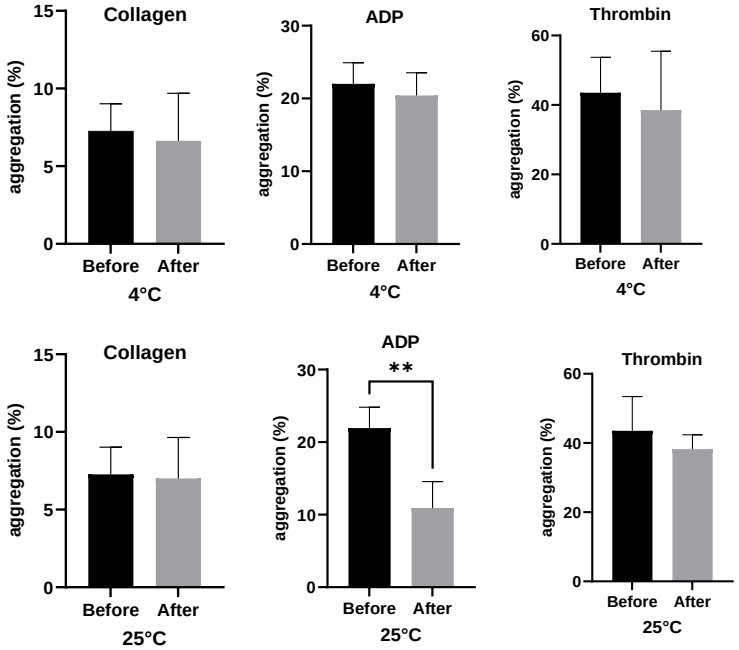

Figure 7. **Platelet aggregation after one-month storage at RT and 4°C:** The results indicated that platelet aggregation remained largely unchanged after one month of storage at 4°C (p > 0.05 for all the agonists). The platelets stored at RT showed a decline in ADP-induced aggregation (t = 20.57, df = 2, p = 0.002) ** P < 0.01

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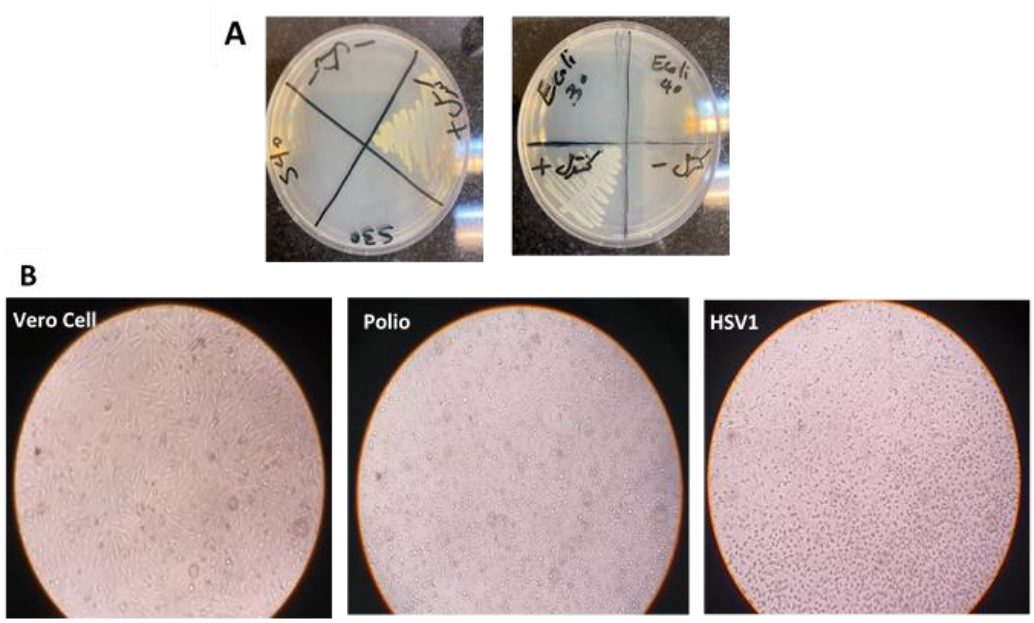

Figure 8. **Pathogen culture and Gamma radiation effects:** (A) Bacterial cultures, including negative and positive controls as well as the lyophilized group treated with 30 kGy and 40 kGy gamma radiation showed no detectable growth. (B) The Vero cell cultures treated with Gamma-irradiated samples were compared to the positive controls for Poliovirus and HSV-1, demonstrating effective pathogen inactivation.

Discussion

Over the years, significant efforts have been made to develop methods of preserving platelets through freeze-drying. Earlier studies highlighted the challenges of preserving platelet integrity during the process. Subsequent attempts introduced innovative cross-linking techniques and additives, which enhanced stability but led to a notable reduction in platelet function (17). More recently, advancements in lyophilized platelet products have been promising, particularly with the use of new stabilizers (10). This research indicates that freeze-dried platelets may retain some metabolic activity. The aim of the current study was to assess the coagulation properties of trehalose- and sucrose-stabilized platelets in vitro. As it was found, trehalose at 60 mM was the most effective in maintaining platelet count, mitigating platelet activation during the lyophilization process, and enhancing platelet aggregation in response to agonists like thrombin and ADP. Indeed, trehalose preserves platelet function more effectively, ensuring that platelets remain responsive to physiological stimuli even after lyophilization. In contrast, the sucrose, combination, and control groups experienced significant decreases in platelet counts post-lyophilization. The inhibitory effect observed in the sucrose-treated groups aligns with the results of previous studies that suggest sucrose may not be as effective in preserving platelet functionality. Trehalose and sucrose are extensively used to stabilize biomolecules in various pharmaceutical and medical applications. Trehalose, in particular, is known for enhancing the shelf life of freeze-dried recombinant proteins and nucleic acids, even when stored at elevated temperatures (18). Differential Scanning Calorimetry (DSC) analysis confirmed that the plasma freeze-dried with trehalose remains in a glassy state at room temperature. Storing biomolecules in this

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glassy state helps reduce protein aggregation and oxidative damage over time (19). These protective effects include the replacement of hydrogen bonds with water molecules surrounding biomolecules and the formation of a highly viscous matrix that slows down damaging reactions. Among disaccharides, trehalose is particularly noted for its lyoprotective properties, as it has a strong glycosidic bond that is less prone to hydrolysis compared to the bond in sucrose (20). The glass transition temperature (Tg) of trehalose, which is the temperature below which a protective glassy state forms, is significantly higher than that of other disaccharides such as sucrose or glucose. Additionally, residual sugars from the preservation process can remain in the samples, potentially interfering with specific diagnostic applications. Trehalose is often preferred over glucose or sucrose because it is not typically present in mammalian cells, minimizing the risk of interference (21). Although trehalose-loaded platelets remain responsive to physiological stimuli even after lyophilization, maximum platelet function decreases compared to fresh PRP. Previous investigations into the function of thrombosomes evaluated their mitochondrial viability and found that the lyophilization process would lead to diminished mitochondrial function (22). It is well established that platelet mitochondria are crucial for aggregation and secretion, as they provide the necessary energy for these processes (23). However, this study revealed that the contribution of thrombosomes to clot formation, as assessed by ROTEM, was comparable to that of fresh platelets in most parameters, including clot initiation and propagation, such as clotting time and angle. Nevertheless, maximum clot firmness was significantly reduced in thrombosome-containing mixtures compared to fresh platelets at both normal (250 platelets/nL) and low (100 platelets/nL) concentrations. Consistent with the aggregation results, this reduction is likely due to the absence of energetic, mitochondrial-driven aggregation and clot retraction in thrombosome (10). In another study, cryopreserved platelets exhibited a significantly reduced aggregation response to agonists compared to fresh and liquid-preserved platelets. Despite this finding, cryopreserved platelets generated higher levels of thromboxane upon stimulation than their fresh and liquid-preserved counterparts. Thromboxane A2, a metabolite of arachidonic acid (AA), is known to induce vasoconstriction and promote platelet aggregation (24). However, vitro analyses, although limited, show that thrombosomes have similar surface marker profiles, some thromboelastogram (TEG) values, and size distributions compared to stored platelets (25). Biomedical products are susceptible to contamination by viruses, bacteria, or other microorganisms. However, current diagnostic methods have limitations, particularly in detecting unknown pathogens, and anti-pathogen antibodies may not be effective during the early stages of infection. Moreover, non-enveloped viruses pose a specific challenge due to their small size and resistance to ionizing radiation (26). To mitigate this problem, proteinaceous materials should be subjected to gamma radiation at a high dose (e.g., exceeding 3 KiloGray/hour) while in a lyophilized state, ensuring the preservation of their functional activity (27). Previous studies have demonstrated that lyophilization is effective in maintaining platelet structure, even under high doses of radiation, due to the existence of stabilizers (28). When the microbial contaminant is a non-enveloped virus, an effective dose of gamma radiation typically ranges from a minimum

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of approximately 25, 30, or 35 kGy to a maximum of about 30, 35, 40, 45, 50, or 60 kGy, with the specific upper and lower limits being independently selected, provided that the lower limit is always less than the upper limit (29). The current study aimed to evaluate the pathogen inactivation efficacy of gamma radiation on lyophilized platelets ranging from a lower limit of 30 kGy to an upper limit of 40 kGy in the presence of stabilizers. As the results showed, gamma radiation was highly effective for pathogen inactivation when combined with lyophilization for all the enveloped and non-enveloped viruses. The significant reductions in viral (Polio and HSV) and bacterial (S. epidermidis and E. coli) loads further validated the efficacy of this approach when applied in a lyophilized state.

Conclusion

The preservation of platelets through lyophilization has undergone significant advancements with trehalose emerging as a superior cryoprotectant. Compared to sucrose, trehalose demonstrates a notable ability to preserve platelet function, reduce platelet activation, and maintain aggregation responses after lyophilization. This effectiveness is attributed to its higher glass transition temperature and robust glycosidic bond, which help maintain platelet integrity and functionality. Furthermore, the combination of gamma irradiation with lyophilization has proved to be highly effective for pathogen inactivation, significantly reducing viral and bacterial loads. This validates its potential for producing safe and reliable biomedical products. However, challenges persist, particularly in optimizing the balance between stability and functionality to enhance the overall efficacy of lyophilized and sterilized platelet products.

Ethical Considerations

This study was approved by the Research Ethics Committee of Higher Institute of Research and Medical Education of Blood Transfusion (IR.TMI.REC.1401.009). Informed consent was obtained from the individual blood donors.

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Authors' Contributions

V.T. and Z.S. and M.J. conceived the study, V.T. performed the study and wrote the manuscript, V.T. and Z.S. analyzed the data, and M.R.D revised the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interests regarding this research.

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