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RESEARCH ARTICLE

# TREHALOSE CAN IMPROVE PLATELET AGGREGATION VERSUS COLLAGEN AGONIST DURING LYOPHILIZED PLATELET PREPARATION

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**ABSTRACT**

Due to short shelf life of platelet concentrate 3-5 days, long-term preservation of human platelets will greatly reduce the risk of their shortage. Lyophilization has been proved feasible approach for this purpose. We want to show that platelets can be preserved by freeze-drying them with loading buffer containing trehalose, a sugar found at high concentrations in organisms that naturally survive drying. Washed platelet samples in a concentration of 0.8-1x10 $^{9}$ plt/mL in loading buffers containing trehalose at various concentrations were incubated at 37°C for 4 h. Platelets were lyophilized with optimized concentration of trehalose 40 mmol/L. The aggregation response of trehalose-loaded platelets was tested against collagen agonist (2.0 µg/ml). Our results showed that aggregation of PRP, resuspended platelet samples in loading buffers containing 10, 20, 30 and 40 mmol/L trehalose and rehydrated lyophilized platelet containing 40 mmol/L trehalose were measured versus collagen agonist by aggregometry instrument with the activity of 7.7%, 22.3%, 45.9%, 71.4% and 35.3% respectively. However, when the platelets lyophilized without trehalose, most of the platelets disintegrated during rehydration step, some fused with adjacent cells and formed an insoluble clump. We concluded that trehalose at the concentration of 40 mmol/L can improve platelet aggregation activity versus collagen agonist in the loading buffer or dried state.

**Keywords:** Trehalose; collagen; lyophilized platelet; platelet aggregation

## INTRODUCTION

Blood transfusion centers are under considerable pressure to produce platelet concentrates for transfusion. Platelet-rich plasma concentrates are stored in blood bags at 22°C, with a shelf life limited to 3-5 days. As a result, these chronic shortages have made investigators to find alternatives to produce lyophilized platelet substitutes[1-6]. Thus, prolonging the shelf life of the platelets would be valuable contribution. In the early 1980s, studies indicated that biomolecules and molecular complexes such as membranes and proteins can be stabilized in the presence of sugars in the dry state and trehalose sugar in comparison with the other sugars were clearly more effective[7]. In 2001, Wolkers et al.freeze-dried platelets and made the recovery rate of 85%, with the protection of trehalose[8]. And they developed a freeze-drying method of platelets of large volume[9,10]. In 2007 Zhou et al. optimized the freeze drying protocol of human platelets with respect to lyoprotectant, cooling rate and cell concentration[11]. Application of protective carbohydrates for preservation of platelets and lipid membranes during lyophilization in responding to environmental stresses such as cold, freezing and drying have been previously studied[12-16]. We want to know in this research that trehalose is particularly effective at stabilizing platelet particles at the concentration of 40 mmol/L and can show better aggregation property versus collagen agonist in comparison with lower trehalose concentrations.

## MATERIALS AND METHODS

Whole blood was obtained from Vesal Blood Transfusion Center (Vesal,Tehran) and then Platelet Rich Plasma (PRP) was prepared by centrifugation (2050 g, 4 min) and aggregation test versus collagen agonist was performed as soon as possible. PRP was centrifuged for 5 min at 500 g to remove leukocytes. The supernatant containing platelet was washed (600 g, 10 min) with washing buffer (100 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L EGTA, 10 mmol/L Imidazol, 10 µg/mL PGE1, pH:6.8). Washed platelet samples in a concentration of 0.8-1x10 $^{9}$ plt/mL in loading buffers (washing buffer in the presence of 10, 20, 30 and 40 mmol/L trehalose) were incubated at 37°C for 4 h. The samples were stirred every 1 h during incubation. At the end of incubation period, aggregation test were carried out on the samples versus collagen agonist (2.0 µg/ml). Platelets in loading buffer with 40 mmol/L trehalose were pelleted and resuspended in lyophilization buffer (9.5 mmol/L Hepes,142.5 mmol/L NaCl, 4.8 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 30 mmol/L Trehalose, 1% human serum albumin, pH:6.8). Platelet concentrates were transferred in freezer -80C° with freezing rate -1C°/min for 90 minutes. After freezing, the frozen platelet solution were transferred to lyophilizator (VaCo 5-II, Zirbus Technology) under100 mili Torr vaccum pressure for 16 h. Freeze dried platelet concentrates were prehydrated for approximately 2 hours in a closed box with moisture-saturated air at 37°C. Vials containing freeze

 

dried platelet rehydrated in 4 ml platelet poor plasma/water (2/1v/v). For aggregation test platelet suspension were transferred to aggregation cuvettes with a magnetic stirrer and response of the platelet to the collagen agonist (2.0 µg/ml) was measured by Helena aggrigometer under stirring condition within 5 min.

## RESULTS

The aggregation of PRP, resuspended platelet samples in loading buffers containing 10, 20, 30 and 40 mmol/L trehalose and rehydrated lyophilized platelet containing 40 mmol/L trehalose were measured versus collagen agonist by aggregometry instrument with the activity of 7.7%, 22.3%, 45.9%, 71.4% and 35.3% respectively that were summarized in Table I.

Table I: Platelet aggregation test results of platelet rich plasma and resuspended platelet samples in loading buffer containing different concentration of trehalose versus collagen (2.0 µg/ml)

| Item | Trehalose concentration (mmol/L) | Aggregation activity (%) |
|---|---|---|
| Platelet rich plasma | 0 | 69.1 |
| Platelets in loading buffer * | 10 | 7.7 |
| Platelets in loading buffer | 20 | 22.3 |
| Platelets in loading buffer | 30 | 45.9 |
| Platelets in loading buffer | 40 | 71.4 |
| Rehydrated lyophilized platelet | 40 | 35.3 |

**Platelets were resuspended in loading buffer containing trehalose and incubated for 4 h at 37°C.*

Clot formation at 37°C was clearly observed within 5 min after addition of collagen agonist (2.0 µg/ml) to rehydrated lyophilized platelets. Furthermore, when the supernatant was measured with the Coulter counter, we found that no platelets were left in suspension. On the other hand, when the platelets lyophilized without trehalose, most of the platelets disintegrated during rehydration step, but the small number were alive, most had fused with adjacent cells, forming an insoluble clump.

## DISCUSSION

It is obvious that if human platelets have been successfully freeze-dried at the presence of optimized trehalose concentration, they would have several advantages over the traditional methods[17] : (1) lyophilized platelets could be stored at room temperature for several months without any cryo-equipments; (2) lyophilized platelets could be reconstituted quickly at the point of use, which is especially important in emergency; (3) lyophilized platelets could be shipped and transported readily.

Introducing trehalose into the cytosol of human platelets with endocytosis pathway at higher temperature (above 25°C) which was discovered as a simple method by Wolkers et al[8]. In our study this simple method was used and platelets were loaded in the presence of 40 mM external trehalose at 37°C for 4 h. With this method trehalose is rapidly taken up by human platelets with loading efficiencies of 50% or greater[8]. Our findings showed that the effect of trehalose on platelet aggregation induced by collagen appears to follow a concentration dependent pattern. Furthermore, platelet aggregation response of loading buffer sample containing trehalose 40 mmol/L was better than fresh platelet rich plasma sample (Table I). Our rehydrated lyophilized platelet indicated full collagen-induced platelet aggregation due to complete clot formation within 5 min. All the rehydrated platelets participated in platelet aggregates formation because we didn't observe any platelet in supernatant of cuvettes after aggregation tests. In addition collagen as a strong agonist shows no primary wave of aggregation and depends on intact membrane receptors, membrane phospholipase pathway integrity and normal cyclooxygenase and thromboxane pathway function. In our experiment the rehydration condition for lyophilized human platelets is optimized from the aspect of prehydration duration and rehydration solution according to previous studies[18,19]. It seems that freeze-dried platelets can retain their structural integrity after rehydration, but proper functioning of these lyophilized platelets in hemostasis as a platelet substitute is questionable and required to be more investigated.

 

## CONCLUSION

The rehydrated platelets showed acceptable response versus collagen agonist, although it was strongly attenuated in comparison with fresh platelet rich plasma.

In general, our study indicated that trehalose at the concentration of 40 mmol/L can improve platelet aggregation activity versus collagen agonist in the loading buffer or dried state. However, trehalose loading efficiency at various concentrations during freezing, drying and rehydration steps needs to be further investigated.

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