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Experimental study on rehydration conditions of freeze-dried platelets*

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Abstract: A rehydration process for freeze-dried human platelets was studied on 1 ml of samples. The effects of prehydration duration, prehydration temperature, and rehydration solution on the recovery rate, mean platelet volume (MPV), and platelet distribution width (PDW) were investigated. The mass changes during the prehydration process were also studied. Three prehydration durations: 0, 1.5, and 3.5 h, and two rehydration solutions: platelet-poor plasma and phosphate-buffered saline (PBS), were tested. It was found that: (1) the prehydration was of significance; (2) 1.5 h of prehydration had better effects than 3.5 h of prehydration; (3) as a rehydration solution, the platelet-poor plasma behaved better than the PBS. The impacts of prehydration duration and temperature on the results were studied. There was almost no difference between 35 and 37 °C. Among all the prehydration durations tested, 15, 30, 60, 90, and 120 min, the best result was achieved with the time duration of 15 min. The weights of prehydrated platelets at the end of each test were measured and the water contents were calculated. After 15 min of prehydration, the water contents in the samples were about (4.8±0.01)% and (5.27±0.29)% (w/w) corresponding to the conditions of 35 and 37 °C, respectively. These results will be helpful for further studies on the freeze-drying of mammalian cells.

Key words: Freeze-drying, Platelets, Rehydration, Prehydration, Rehydration solution
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INTRODUCTION

Fresh autologous platelet-rich plasma (PRP) has been increasingly used over the last decade for enhancing wound healing (Marx *et al*., 1998; Kim *et al*., 2002; Bhanot and Alex, 2002). Platelets are generally stored in blood banks at room temperature (22 °C), with a shelf-life limited to 5 d by government regulations due to the increased risk of bacterial contamination. Exposing platelets to temperatures below 20 °C can reduce bacterial proliferation but may induce irreversible changes in their physiology including conversion from discoid to spherical shape, activation of numerous filopodia, and release of α-granules (Tablin *et al*., 2001). Cryopreservation of platelets using dimethyl sulfoxide has gained some successes in addressing this problem, but thawed platelets must be washed prior to transfusion to remove the potentially harmful cryoprotectant, which causes more damages to the cells (Bock *et al*., 1995). As a consequence, it is expensive to process and maintain platelets. Freeze-drying is an ideal alternative in principle for long-term preservation of platelets in blood banks. It has several advantages over the traditional methods (Liu *et al*., 2007): (1) freeze-dried platelets could be stored at room temperature for an extended period of time without the need of cooling equipments; (2) freeze-dried platelets could be reconstituted quickly by rehydration at the point of use, which is convenient and important in emergency; (3) freeze-dried platelets

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have a low density that can be readily shipped and transported. The rehydrated, lyophilized platelet is being developed as a hemostatic infusion agent for the control of active bleeding recently (Bode *et al*., 2008). Read *et al*.(1995) lyophilized the paraformaldehyde-fixed platelets. Those platelets indeed seemed to retain their structural integrity after rehydration, but the proper functioning of those chemically modified platelets in hemostasis was questionable. Wolkers *et al*.(2001) loaded trehalose into platelets cells and successfully freeze-dried the platelets. Zhou *et al*.(2007) optimized the protocol of freeze-drying platelets with respect to protectant, cooling rate and cell concentration. Up to now, most of these studies have focused on the prophase processes including preparation, freezing and drying. However, the primary research has shown that the upper process, rehydration, is also very important. Han *et al*.(2004) tested different reconstitution solutions at different temperatures, and found that the survival of lyophilized erythrocytes was dependent on colloidal osmotic pressure more than on crystalloid osmotic pressure. Shu *et al*.(2007) rehydrated red blood cells at different temperatures and got the highest cell recovery rate at 37 °C. They suggested that membrane phase transitions and extreme volume response during rehydration might damage the cells. Wolkers *et al*.(2001) found that prehydration had good effects on the morphology of rehydrated platelets. However, they did not provide the way of how to choose the prehydration duration for other freeze-drying application.

In this study, the different prehydration durations, prehydration temperatures, and rehydration solutions were considered for the rehydration of 1 ml of freeze-dried human platelets. The recovery rate, mean platelet volume (MPV), and platelet distribution width (PDW) of the rehydrated platelets were measured as viability standards. The water contents of the samples at several time points of the prehydration process were also obtained. They might have some relations with the recovery rate.

MATERIALS AND METHODS

Platelets preparation

Platelet concentrates (PRP) were obtained from healthy volunteers, provided by the Blood Center of Zhejiang Province, China. They were stored at 22 °C on a flatbed shaker overnight before the experiments. PRP was centrifuged and washed twice (at 2000 r/min for 10 min) with physiological saline.

Incubation for trehalose loading

Washed platelets were resuspended in a loading buffer (100 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L ethylene glycol tetraacetic acid (EGTA), 10 mmol/L imidazole, 50 mmol/L trehalose, and pH 6.8). The platelet solution with a concentration of about 1×10^9 ml^{-1} was incubated in a 37 °C water bath for 4 h to load trehalose into the platelets. During incubation, the samples were gently stirred constantly.

Preparation of lyophilization suspensions

After incubation, the platelet samples were collected by centrifugation (at 2000 r/min for 3 min). Then the platelets were resuspended in a lyophilization buffer. 9.5 mmol/L N′-a-hydroxythyl-piperazine-N-ethanesulfanic acid (HEPES), 142.5 mmol/L NaCl, 4.8 mmol/L KCl and 1 mmol/L $MgCl_2$ were used as the base solution. The lyoprotectants used in this study include 528.6 mmol/L trehalose and 1% (w/v) BSA. Cell count, the MPV and PDW were measured by a CELL-DYN1700 hemacytometer (Abbott, USA). The cell concentration in lyophilization buffer was about 1×10^9 ml^{-1}.

Freezing

Aliquots of 1 ml platelet suspensions were filled into glass vials (diameter: 22 mm) and frozen in the cryo-refrigeratory at −60 °C for 2 h, with a cooling rate of about 10 °C/min.

Freeze-drying

The freeze-drying was performed in a laboratory-scale freeze-drier (Labconco, USA) with the following four steps: (1) the freeze-drier chest was precooled to −40 °C at a rate of 1.5 °C/min while the cold condenser was cooled to −80 °C; (2) the frozen samples were put onto the shelf of freeze-drier chest, and then started the vacuum pump; (3) after primary drying for 16 h, the shelf was warmed up to 22 °C at a rate of 0.2 °C/min and then kept at that temperature for about 16 h.

During the desiccation process, the vacuum chamber pressure was kept at about 1 Pa. After freeze-drying, the samples were sealed with rubber stoppers automatically and stored at room temperature (22 °C).

Rehydration

Two solutions, 1 ml of platelet-poor plasma:water (1:1, v/v) mixture and 1 ml of phosphate-buffered saline (PBS) buffer:water (3:1, v/v) mixture, were used during the rehydration test. The PBS buffer was composed of 100 mmol/L NaCl, 9.4 mmol/L Na_2HPO_4, and 0.6 mmol/L KH_2PO_4 (pH 6.8). Some samples of lyophilized platelets were prehydrated in a vessel with water saturated air at 35 °C (equivalent to a partial pressure of water vapor of 5.9 kPa) for 3.5 h, and some were prehydrated for 1.5 h. They were compared with the directly rehydrated lyophilized samples.

After prehydration, each sample was rehydrated in 1 ml of platelet-poor plasma:water (1:1, v/v) mixture or in 1 ml of PBS buffer:water (3:1, v/v) mixture at room temperature (22 °C), followed by gentle shaking until the sample was completely dissolved in the rehydration solution.

Compositive ortho-experiments were conducted to study the effects of the above-mentioned factors on the cells recovery rate.

After the compositive ortho-experiments, the impacts of prehydration duration and temperature were investigated further. The preparation and freeze-drying stages were the same as the ortho-experiments. Samples of lyophilized platelets were prehydrated in a vessel with water saturated air at 35 or 37 °C (equivalent to a partial pressure of water vapor of 5.9 kPa) for 15, 30, 60, 90, 120, 150 or 210 min, respectively. After prehydration, each sample was rehydrated in 1 ml of platelet-poor plasma:water (1:1, v/v) mixture at room temperature (22 °C), followed by gentle shaking until the sample was completely dissolved in the rehydration solution.

Water contents of prehydrated platelets

An electronic-analytical balance (Mettler AE 200, with the precision of 0.1 mg, Switzerland) was used to measure the weights of samples in order to obtain the water contents of prehyrated platelets. First, the empty vials with labels were measured (W_e). After freeze-drying, the vials with lyophilized platelets were measured (W_d). Then, they were moved into a vessel for prehydration. After prehydration, the vials with prehydrated samples were measured (W_p). The water contents of freeze-dried platelets before prehydration (C_d) were measured by thermo-gravimetry using Q600 Simultaneous DSC-TGA (TA Instruments, USA). About 5 mg of powder were placed on the TG pan and heated from room temperature (22 °C) to 120 °C at a heating rate of 10 °C/min. C_d was the ratio of the lost water weight to the initial sample weight.

The water contents (C_w, g water/g wet basis) of prehydrated platelets were calculated as $C_w=[(W_d-W_e)\times C_d+(W_p-W_d)]/(W_p-W_e)\times 100\%$.

Recovery

The numerical recovery of lyophilized and pre-rehydrated platelets (R_p) was determined by counting platelets with a CELL-DYN1700 hemacytometer (Abbott, USA) before drying and after rehydration. And the MPV and the PDW after rehydration were measured by the same hemacytometer.

$$R_p=\frac{N_a}{N_b}\times 100\%,$$

where N_a is the number of platelets after free-drying and rehydration, N_b is the number of platelets before freeze-drying.

Aggregation test

Fresh and rehydrated platelet suspension (400 μl) were both moved to the aggregation cuvettes with a stirring bar inside and warmed to 37 °C. Then, thrombin was added into the platelet suspension in turn to a final thrombin concentration of 1 μmol/ml. Aggregation traces of the fresh and rehydrated platelets were both recorded with an aggregometer (Chrono-Log, USA).

RESULTS

Haematology results

Table 1 shows the compositive ortho-experiment results for different rehydration solutions and prehydration durations. Prehydration was carried out

at 35 °C. It can be seen that the samples rehydrated with plasma:water (1:1, v/v) mixture have a higher recovery rate than those rehydrated with PBS:water (3:1, v/v) mixture. Samples prehydrated for 1.5 h have the highest value of platelets recovery and those for 3.5 h have the lowest one. Samples directly rehydrated also have relatively high recovery rates over 80%. The groups of recovery data for platelets rehydrated with plasma:water (1:1, v/v) mixture were analyzed by ANOVA. As a result, a statistically prominent difference exists between the prehydration durations of 1.5 and 3.5 h, and little difference exists between the prehydration durations of 1.5 and 0 h.

Table 1 Compositive ortho-experiments and results at 35 °C (*n*=4)

No.	Rehydration solution	Prehydration duration (h)	Platelets recovery (%)
1	Plasma:water (1:1, v/v)	3.5	64±0.4[b]
2	Plasma:water (1:1, v/v)	1.5	86±8.6[a,c]
3	Plasma:water (1:1, v/v)	0	83±3.7[a,d]
4	PBS:water (3:1, v/v)	3.5	70±5.8
5	PBS:water (3:1, v/v)	1.5	81±6.5[c]
6	PBS:water (3:1, v/v)	0	75±5.4[d]

[a] P=0.59>0.05; [b] P=0.04<0.05; [c] P=0.49>0.05; [d] P=0.02<0.05

Table 2 shows the recovery rates of further experiments on prehydration temperature and duration. At 35 °C, as prehydration duration increases from 15 to 120 min, platelets recovery decreases from 92% to 74%. The difference between 15-min group and 60-min group is statistically significant. Similar trend can be observed for experiments carried out at 37 °C. Little statistical difference exists between prehydration temperature of 35 and 37 °C.

Figs.1a~1b show the changes of MPV and PDW values for various prehydration durations at 35 °C. It can be seen that the freeze-dried platelets prehydrated for 15 min have the smallest average MPV and the lowest average PDW, close to the MPV for fresh human platelets, (11.81±0.90) fl (Liu and Lv, 2007) and the PDW for fresh human platelets, (17.02±1.10)% (Chen, 2006). Further ANOVA analysis demonstrates that significant MPV and PDW differences exist between 15-min group and other groups. Therefore, 15 min or less may be the most appropriate prehydration duration for 1 ml freeze-dried human platelet samples.

Table 2 Platelets recovery rehydrated with plasma/water (*n*=4)

Prehydration duration (min)	Prehydration temperature (°C)	Platelets recovery (%)
0	35	85±5.3[a]
	37	–
15	35	92±7.8[a,b,c]
	37	95±9.4
30	35	87±3.8[b]
	37	86±10.4
60	35	80±5.3[c]
	37	79±6.9
90	35	79±2.4
	37	78±6.9
120	35	74±3.2
	37	77±8.2

[a] P=0.25>0.05; [b] P=0.46>0.05; [c] P=0.03<0.05

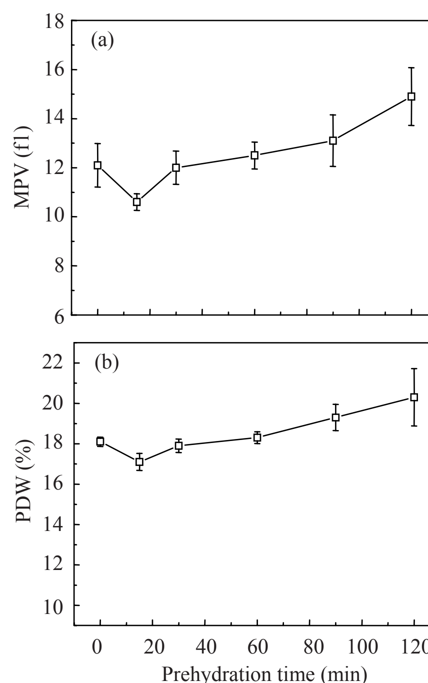
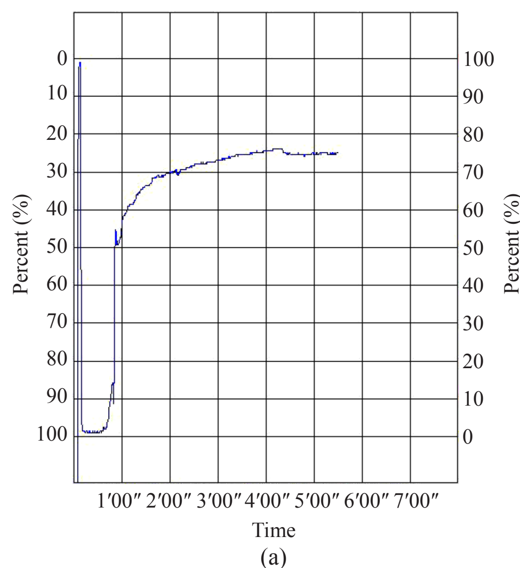


Fig.1 MPV (a) and PDW (b) of rehydrated platelets (*n*=4)

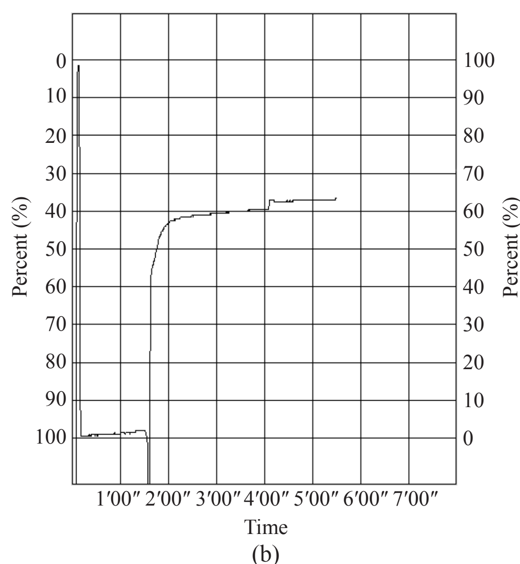
Response of freeze-dried platelets to thrombin

The response of fresh and rehydrated freeze-dried platelets to thrombin was investigated by aggregometry. The aggregation trace was shown in Figs.2a~2b. The maximum aggregation percentage to thrombin (1 μmol/ml) for rehydrated freeze-dried platelets was 84% of that for the fresh ones, and the

responsive time was both in a second. These results demonstrated that these freeze-dried platelets still responded to thrombin (1 μmol/ml). It seems that freeze-dried human platelets maintain a good aggregation function.



(a)



(b)

Fig.2 Aggregation traces of platelets. (a) For the fresh platelets; (b) For the prehydrated freeze-dried platelets

Changes of water content

The water content of freeze-dried samples before prehydration (C_d) was about 2.5%. Fig.3 shows the water contents (C_w) of prehydrated samples. It can be seen that they increase with the prehydation time at both temperatures of 35 and 37 °C. For the same prehydration time, the water contents observed at 37 °C is a little higher than that at 35 °C. For example, after prehydration for 15 min at 35 °C, the average water content is (4.8±0.01)% (w/w), while at 37 °C the average water content is (5.27±0.29)% (w/w). After prehydration for 150 min at 35 °C, the average water content can reach (12.4±0.32)% (w/w), near equilibrium.

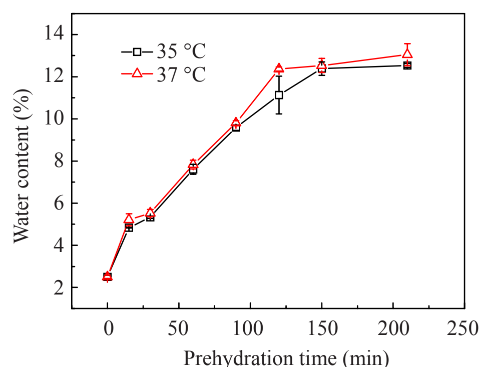


Fig.3 Water contents of prehydrated samples (*n*=4)

DICUSSION

Wolkers *et al*.(2002) proposed to use prehydration to improve cell recovery rate after rehydration. In their study, 1 ml platelet lyophilysates were prehydrated in a closed box with water saturated air at 37 °C (equivalent to a partial pressure of water vapor of 6.3 kPa) for 3.5 h, while samples in bags (80 ml) were prehydrated for 1.5 h. Then, the 1 ml samples were rehydrated with 4 ml rehydation solution, while the 80 ml samples were rehydrated with 80 ml rehydation solution. They observed that the morphological changes of platelets were greatly reduced by prehydration of the lyophilysate. It was believed that the prehydration affects the physical properties of the cell membranes and prevents the membrane fusion and leakage of cytoplasmic components.

It is a pity that Wolkers *et al*. (2002) did not give the guidelines about how to select the prehydration temperature and duration. For other type of cells, the prehydration may also be helpful, but how this process should be carried out remains unclear. Therefore, it is important for further investigating human platelets in order to elucidate more information about prehydration.

Here freeze-dried human platelets were

prehydrated at 35 and 37 °C. Both temperatures were found beneficial to the survival of platelets with little difference statistically. This phenomenon may be explained by the phase transition of cell membrane. The phase transition temperature of human platelet membrane is between 15 and 18 °C (Tablin *et al*., 1996). And from the results of Shu *et al*.(2007)'s experiments, there are two phase transition temperatures of the dried membranes of red blood cells: 10 °C and 32~34 °C. The two temperatures tried here are higher than the phase transition temperature. Therefore, phase transition would not happen.

A series of prehydration durations, from 15 to 210 min, were tried. By considering the values of cell survival rate, MPV and PDW together, a 15 min of duration was appropriate. Since the duration around 15 min is not tested in this study, the optimal duration may exist other than 15 min. The existence of optimal duration may be due to the state of water molecules at different water content. At low water content, the water molecules absorbed by the dry sample will act as bound water, weak or strong. When the water content is higher than a certain value, the water molecules absorbed will act as free water. When liquid water appears in the dry sample, high concentration solution (HCS) will appear at the same time. As prehydration goes on, HCS may be diluted gradually. HCS will bring damage to cells, as illustrated by lots of literature (Acker *et al*., 2002). Therefore, the measurements of water contents of the samples after various prehydration durations are of significance. For the 15 min duration, the average water content was about 5%. In order to verify the point stated above, further studies such as calorimetric measurement are needed.

For large or thick samples, the prehydration process can be physically viewed as two continuous steps. Firstly, water molecules are diffusing into the sample and then absorbed as bound water or accumulated as free liquid. It should be noted that the sample volume in the present study is very small (1 ml), and the thickness of the dried sample is thin (about 2.6 mm). Water vapor diffusion in the samples is relatively quick. For bulk or thick samples, the dynamics of diffusion has to be considered.

On rehydration solution, Wolkers *et al*.(2002) tested PBS:water (3:1, v/v) mixture and PBS:plasma:water (1:2:1, v/v/v) mixture, but did not compare the effects of these two kinds of solutions. Here, we tried to use plasma:water (1:1, v/v) mixture and PBS:water (3:1, v/v) mixture as rehydration solution. According to the data of recovery rate, these two solutions have no statistically difference. However, for the case of direct rehydration, plasma:water (1:1, v/v) mixture behaves better than PBS:water (3:1, v/v) mixture, which may be due to the fact that plasma is closer to the original solution environment where platelets exist.

In fact, it is indicated that in recovery, the prehydration makes little significance contrast to the direct rehydration, statistically. However, as Wolkers *et al*.(2002) mentioned, the percent recoveries in the non-prehydrated samples were probably over-estimated. They went on to show that after prehydration, the average optical density (OD) as measured on an absorbance spectrophotometer, increased by a large margin, and much more closely approximated the OD of fresh cells. Here we measured the MPV and PDW values of rehydrated freeze-dried platelet samples with or without prehydration, and resulted that the MPV and PDW values of rehydrated freeze-dried platelet samples with prehydration for 15 min were more closely approximated the MPV and PDW values of fresh cells.

Since the circumstances under which lyophilized platelets would be needed (e.g., combat, excessive bleeding) are likely to necessitate a rapid response, and the direct rehydrated freeze-dried platelets may remain some functions, some may consider the prehydration making no sense. However, as the results of our or some others' studies (Wolkers *et al*., 2002), the prehydration makes beneficial to the freeze-dried platelets. As the theory of this process to the platelets is still unclear, it is worth to be researched further.

CONCLUSION

Compositive experiments were conducted for the rehydration of freeze-dried platelets. The results are as follows: (1) It is advantageous to rehydrate the freeze-dried platelets with a prehydration, and a 15 min of prehydration may be the best duration for 1 ml of lyophilized platelets sample; (2) The prehydration

can be implemented at 35 and 37 °C; (3) The plasma:water (1:1, v/v) mixture and PBS:water (3:1, v/v) mixture are the suitable rehydration solutions if a prehydration is conducted. Although there are theories for explaining the influence of temperature, the detailed mechanisms of the prehydration is still not clear. Further study is needed to understand the process.

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