Effect of Nitric Oxide Donor on Metabolism of Apheresis Platelets

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Abstract The aim of this study is to investigate the effects of a nitric oxide (NO) donor S-nitrosoglutathione (GSNO) on the metabolism and improvement of preservation quality in apheresis platelets. A GSNO solution with a certain concentration was added into fresh apheresis platelets, and the parameters associated with platelet morphology, metabolism and function were temporally monitored for 7 days. The results showed that the NO level in GSNO group were remarkably higher than those in the control group during the whole storage stage. No significant morphology or function difference was observed between the control and GSNO groups such as Platelet count, platelet distribution width, mean platelet volume and mitochondrial metabolic activity. But in metabolism there are something differences from morphology data: $p\text{CO}_2$, $p\text{O}_2$, CHCO_3^- were also found to have no clear difference between the control and GSNO groups; the lactic acid content, sugar consumption and Lactate dehydrogenase activity in the GSNO group were lower than that in the control group at some time point; and pH values in the

GSNO group were higher than the control group. Our study discovered that the NO donor GSNO can reduce the metabolism and maintain the cellular characteristics of platelets in vitro during the platelet storage period.

Keywords GSNO · Nitric oxide donor · Platelet storage lesion

Introduction

Because of irreplaceable hemostatic effect, platelet transfusion is widely used in clinic. Platelets participate not only in physiological hemostasis and coagulation, but also in plenty of pathophysiology processes such as thrombosis and hemorrhagic disease, inflammation, tumors, and metabolic diseases. To achieve effective platelet transfusion, platelet preservation quality before transfusion is particularly important.

During the platelet storage, a series of changes involving morphology, biochemistry, and functions of platelets usually occur, which is known collectively as platelet storage lesion (PSL) [1–3]. The changes result in both impairment of platelet quality and shorter storage life of platelets. In addition, PSL also affects hemostatic function and the survival of platelets in the recipient after transfusion [4].

A number of research efforts have been carried out to explore the mechanism of PSL and identify a solution to promote a more effective platelet transfusion. But, the specific mechanism of PSL is multifactorial and not yet fully understood. However, metabolic disturbance and inadequate metabolic support play an important role in the loss of platelet viability in the PSL [5–7]. The preservation quality of platelets is closely related to pH, as a pH < 6.0 greatly reduces platelet viability [8, 9]. During storage, the

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oxidation of long chain fatty acids impaired which leading to increase in glucose consumption, production of lactate, and a decrease in pH, maybe one deep reason for PSL [3, 10]. The pH fall is also closely related to bicarbonate depletion, and bicarbonate depletion maybe generates an increase in lactate concentration [11]. The progressive morphological changes in platelet during storage cannot be ignored [7], a change in the shape of the platelet is a manifestation of cell apoptosis, which is responsible more detailedly for exposure of phosphatidyl serine and generation of microvesiculation [12, 13].

Developing a method to reduce PSL to ensure platelet quality is of great interest. The rate of development of storage lesions can be postponed by improving platelet blood bags, storage media, and processing [8, 9]. The inclusion of some anti-platelet agents and drugs during processing and storage serves to slow the activation process, resulting in more quiescent cells [14].

Nitric oxide (NO) is an important molecule in vivo, and usually plays as a messenger or effector participating in multiple biological activities such as regulating several physiological and pathological processes. Nitric oxide is an anti-platelet agent with the ability to suppress platelet aggregation and activation in vitro and in vivo [15], which provides a new approach to improve the quality of platelets in vitro. At present, limited reports are available on the impact of NO on the metabolism and quality of platelet during storage time. In this research, S-nitrosoglutathione (GSNO), a NO donor, was added into apheresis platelets, and the parameters associated with platelet metabolism were dynamically monitored by simulating platelet storage in standard blood bank. The aim of this study was to investigate whether the addition of GSNO to stored platelets can enhance the function of platelets, reduce the PSL and metabolism, and improve platelet preservation quality.

Materials and Methods

Samples

Apheresis platelets were collected from 30 healthy blood donors in Ningbo Central Blood Station, Zhejiang Province, China. All donors did not take aspirin or other drugs affecting platelet function within 2 weeks prior to the donation. Written informed consent was obtained from all donors, and this study was approved by the local ethical committee.

Each sample included 150 ml platelet concentrate (PC) with more than 1×10^9 PLT/ml. On the day of PCs collection (day 0), each PCs in the bag was equally divided into two parts using a connecting device instrument (TSCD-II, Terumo Sterile Tubing Welder, Japan) and a

digital balance (Sartorius, Germany). GSNO powder (Sigma-Aldrich, Germany) was dissolved in phosphate buffer saline (PBS) with a store concentration at 1 mM and filtered using 0.22 μ m filters (Millipore, USA). The final concentration of 100 μ M was chosen based on our pilot study and Sener's protocol [16]. GSNO solution (1 mM) was added to one group of bags which made GSNO group, with a volume ratio at 1:9 (1 mM GSNO:PCs) using a sterile syringe by aseptic infusion. Equal volume of sterile normal saline was added to the other group of bags to make the control group. Both groups of PCs were kept in a platelet incubator at 22 ± 2 °C and agitated constantly for 7 days. All parameters were tested on days 1, 3, 5 and 7 of storage.

NO Assay

The NO levels in PCs were measured using a NO assay kit (Jiancheng Biotechnology, Nanjing, China). Following the manufacturer's protocol, the reduction of nitrate to nitrite was done after 15 min at 37 °C. By centrifuging the mixture for 10 min at 4000 rpm, the supernatant was used for assessment of nitrite levels, which reflected the free NO content. The results were given as μ mol/L.

Routine Platelet Test

Platelet counts (PLTs), mean platelet volume (MPV), and platelet distribution width (PDW) were measured using an automated blood counter (Sysmexkx 2100, Sysmex Corporation, Hyogo, Japan).

MTS Assay

In order to measure the mitochondrial metabolic activity of stored PCs, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, USA) was used. The MTS assay [17] is a suitable indicator of mitochondrial activity. According to the manufacturer's protocol: first, stored PCs were diluted in PBS to a concentration of 3×10^5 platelets/ μ l; second, 100 μ l of diluted PCs were incubated with 10 μ l of MTS at 37 °C in a water bath for 4 h; third, the absorbance value was measured at 490 nm by the spectrophotometer.

Blood Gas Analysis and Lactate Dehydrogenase Activity

To avoid exposure to air, sampling was not done using a sample bag, but by direct insertion of a needle in the bag through a sampling site coupler, followed by slow aspiration and immediate analysis on the blood gas analyzer. Glucose, lactate, pH, amount of HCO_3^- , and blood gases

of pO_2 and pCO_2 were immediately determined using routine blood gas equipment (ABL 800; Radiometer, Copenhagen, Denmark). The pH of all samples was measured at 37 °C.

Lactate dehydrogenase activity (LDH) was measured by colorimetric methods with a Chemistry Analyzer (AU5400, Japan).

Statistical Methods

Data were presented as mean $\pm$ standard deviation. GraphPad prism 6 statistical software (GraphPad Software, La Jolla, CA, USA) was used for the analysis. The statistical difference between GSNO and control groups was assessed by the Student's *t* test. Given the non-Gaussian distribution of the results, non-parametric Kruskal–Wallis ANOVA was applied to compare the data from groups [18–20]. A *p* value less than 0.05 was considered statistical significance.

Results

NO Measurement

We examined the NO levels in PCs which indicated the availability of the NO donor by a NO assay kit. The levels of NO in two groups are presented in Fig. 1. In the control group, the concentration of NO increased slightly over time, while the concentration of NO in the GSNO group first increased sharply, than decreased slightly after reaching a peak on day 3. Levels of NO in the GSNO group were markedly higher than those in the control group on during the whole storage stage.

Platelet Count, PDW, MPV and MTS

The levels of platelet count, PDW, and MPV in two groups are presented in Table 1. No significant differences were observed between the control group and the GSNO group at each time point in platelet count, PDW, and MPV. However, differences were observed in PDW and MPV within each group at different time points. Especially when compared with data of the 7 days, there were significant differences at days 1, 3, and 5, respectively.

An MTS kit was used to detect platelet mitochondrial metabolism activity. During the 7 days of storage, the MTS activity of platelets in both groups decreased with time; no significant difference was observed between the control and GSNO groups during storage (Fig. 2a).

Platelet Metabolic Marker Tests

All results of the metabolic parameters in two groups including glucose, lactate, LDH activity, pO_2 , pCO_2 , HCO_3^- , and pH are presented in details in Table 2. The content of lactate increased gradually as storage time increased, in both the control and GSNO groups. The lactic acid content in GSNO group was prominent lower when compared to control group at days 3, 5, and 7, respectively (Fig. 2b). Sugar consumption was slightly lower in the GSNO group than in the control group at days 3 and 7 (Fig. 2c). Additionally, the pH value of the platelets in two groups decreased with time regardless of treatment with GSNO, but remained above 6.8 on day 7, consistent with the pH value requirements at the end of platelet storage. The pH values in the GSNO group were higher than those in the control group at days 1, 3, 5, and 7, but not significant. As seen in Fig. 2d, the LDH activity of GSNO group (163.3 ± 10.3 IU/L) was obviously lower than that of the

Fig. 1 NO levels at different days of storage in GSNO-treated PCs (GSNO) and PCs without GSNO (control), *n* = 9

Table 1 Morphologic indicators of PCs over 7 days of storage

Parameters, day of storage, N = 5	Control	GSNO	<i>p</i> value
PLTs ($\times 10^9/L$)			0.849
0	715.4 $\pm$ 75.4	715.4 $\pm$ 75.4	
1	694.0 $\pm$ 49.0	629.6 $\pm$ 84.9	0.180
3	679.0 $\pm$ 89.5	653.4 $\pm$ 81.2	0.648
5	616.6 $\pm$ 109.7	637.2 $\pm$ 94.8	0.759
7	562.6 $\pm$ 125.9	572.0 $\pm$ 109.1	0.903
PDW			0.965
0	17.12 $\pm$ 0.55	17.12 $\pm$ 0.55	
1	17.08 $\pm$ 0.25	17.22 $\pm$ 0.19	0.349
3	17.06 $\pm$ 0.44	17.36 $\pm$ 0.40	0.293
5	17.36 $\pm$ 0.44	17.38 $\pm$ 0.56	0.951
7	18.22 $\pm$ 0.95	18.18 $\pm$ 0.93	0.948
MPV (fL)			0.982
0	7.50 $\pm$ 0.52	7.50 $\pm$ 0.52	
1	7.32 $\pm$ 0.57	7.48 $\pm$ 0.61	0.678
3	7.40 $\pm$ 0.70	7.46 $\pm$ 0.84	0.905
5	7.60 $\pm$ 0.45	7.56 $\pm$ 0.53	0.901
7	8.30 $\pm$ 0.75	8.12 $\pm$ 0.72	0.710

Fig. 2 Functional and metabolic variation of PCs in two groups at different days of 7 day's storage over 7 days **a** MTS; **b** lactate production; **c** glucose content; **d** LDH activity. * $p < 0.05$, for glucose and lactate test, $n = 5$; for MTS and LDH activity, $n = 6$

control group (205.3 ± 12.7 IU/L) at the last day of storage.

Overall, pCO_2 , pO_2 and $CHCO_3^-$ decreased gradually in the control group and GSNO group over time. The results showed that pCO_2 , pO_2 , and $CHCO_3^-$ did not differ

remarkably between control and GSNO groups during storage, but significant differences were observed between all time points within each group according to ANOVA analysis.

Table 2 Metabolic variables of PCs over 7 days of storage

Parameters, day of storage, N = 6	Control	GSNO	<i>p</i> value
pH _{37°C} ,			0.968
0	7.417 ± 0.161	7.417 ± 0.161	
1	7.455 ± 0.038	7.458 ± 0.048	0.895
3	7.402 ± 0.076	7.422 ± 0.062	0.628
5	7.348 ± 0.077	7.357 ± 0.065	0.843
7	7.202 ± 0.123	7.232 ± 0.120	0.679
pO ₂ (mmHg)			0.986
0	181.5 ± 9.5	181.5 ± 9.5	
1	175.8 ± 7.4	178.8 ± 5.3	0.438
3	172.5 ± 6.1	175.7 ± 3.6	0.299
5	161.8 ± 9.7	162.3 ± 12.1	0.391
7	150.8 ± 16.0	152.3 ± 20.9	0.892
pCO ₂ (mmHg)			0.970
0	35.8 ± 10.0	35.8 ± 10.0	
1	27.0 ± 6.5	23.8 ± 4.7	0.354
3	20.0 ± 5.0	18.0 ± 3.9	0.457
5	17.7 ± 4.2	14.9 ± 3.7	0.261
7	12.8 ± 2.7	11.5 ± 2.7	0.417
cHCO ₃ ⁻ (mmol/L)			0.967
0	19.0 ± 3.4	19.0 ± 3.4	
1	16.8 ± 2.5	15.3 ± 2.8	0.350
3	13.3 ± 3.4	12.0 ± 2.7	0.467
5	10.8 ± 3.0	9.3 ± 2.7	0.377
7	7.0 ± 3.1	5.7 ± 2.8	0.453

Discussion

PSL, a complex process affected by physical, chemical, and metabolic factors, is closely related to the preparation or storage of platelets, which may greatly reduce the quality of platelet components. Researches show that the key way to improve the quality of platelets is to reduce the activation and metabolic rate of platelets as much as possible. Metabolic disorder is one of the leading causes of reduced platelet activity. Platelets derive substantial energy from β -oxidation of fatty acids. However, during storage, the oxidation of long chain fatty acids is impaired by various factors, resulting in an increase in glucose consumption, excessive production of lactic acid, and a decrease in pH [21]. Gulliksson [22] advised that one way of improving the storage quality of platelets is reducing glucose consumption and lactic acid accumulation.

NO can improve platelet function both in vivo and in vitro [23–25], because the platelets in vitro cannot produce sufficient NO to inhibit the activation after withdrawing from endothelial cells which were considered as one important source of NO. GSNO, a NO donor, is involved in the metabolism of NO in platelet cells which is reversible. GSNO is also a type of S-nitrosothiol (RSNO)

which is produced by NO and thiol compounds. In fact, the RSNO content reflects the content of bound form NO in vivo, but the NO content measured in our experiment reflects the level of free-form NO in vivo. The NO data showed that the platelets also produced a certain concentration of NO in vitro without adding GSNO, and the consumption rate of NO is more rapid than the rate of NO release after day 3 no matter treated with GSNO.

We did not observe differences in PLT, MPV, and PDW between GSNO and control group, suggesting that GSNO had no contribution to damage the platelet structure. Furthermore, maintaining of normal platelet structure is also important. It is worth mentioning that in both groups there were significant differences between day 7 and any other days, in terms of MPV and PDW, maybe even pH, indicating that accelerated apoptosis occurred accompanied by platelet morphological and environment changes, consistent with the MTS experimental data. Extending the platelet preservation period may rely more on the breakthrough of platelet apoptosis mechanism research [5, 13]. It is requisite to do more work on inhibition of platelet apoptosis, despite GSNO being able to reduce metabolism and no damage to the cells' morphology. Further experiments will need to be taken to demonstrate

whether GSNO can inhibit the platelet apoptosis. The MTS method is a sensitive assay to detect the metabolic state of cells. Platelets do not contain a nucleus, but they do contain mitochondria. The metabolic activity and function of platelets can be evaluated by testing MTS or MTT activity [26, 27]. Mitochondrial metabolic activity is highly correlated with the oxidation pathway [27], and oxidation can easily lead to PS_L.

The blood gas analysis results indicated that there was a decline in cellular oxygen utilization, and that anaerobic respiration leading to accumulation of lactic acid or other acidic metabolites caused a continuous decline of pH and HCO_3^- . Tynngard et al. [28] concluded that higher consumption of oxygen, reduced glycolysis, and decreased lactate production would improve the viability of platelets. Deyhim et al. [29] studied the effects of L-carnitine on the storage quality of platelets, they proposed that L-carnitine could improve the oxidation pathway of fatty acids by better maintaining HCO_3^- and consuming more oxygen to maintain an optimal pH. Our results coincide with those of Tynngard, as we found the decrease in pO_2 and lower glucose consumption in GSNO group, but differ from those of Deyhim, so we hypothesized that GSNO and L-carnitine may have different mechanism to enhance the viability of platelets, and a blend of GSNO and L-carnitine may complement each other to improve the storage quality of platelets. The lactic acid level and NO measurement results suggested that starting from day 3, free NO released by the NO donor GSNO inhibited lactate accumulation to some extent. Because the release of LDH is related to the integrity of the cell membrane [30], reducing the release of LDH from the cytoplasm and providing an adequate oxygen supply are two important factors in improving the survival and quality of platelets during the storage. Our results showed that at the last day of storage after addition of GSNO, cellular membrane damage was reduced compared with the control group, suggesting that NO plays a role in improving platelet storage quality.

Most current researches have focused on the effects of NO donors on platelets in vivo. For example, NO donors can increase plasma NO levels, inhibit platelet aggregation, and reduce arterial thrombosis in vivo [31]. In a cardiopulmonary bypass model, an external NO polymer, acting as NO donor, prevented platelet consumption and maintained platelet function [32]. The effects of GSNO on platelet preservation in vitro have been mainly focused on the cryopreservation or frozen platelets [33, 34]. The addition of GSNO to frozen platelets can inhibit platelet activation and maintain aggregation activity and platelet function, indicating that GSNO may show potential as a platelet cryoprotectant [34]. According to Wong et al. [35], a certain quantity of NO solution directly injected into the platelets was observed to improve platelet function.

Our work showed that the addition of GSNO can reduce the metabolism and maintain the cellular characteristics of platelets in vitro during the platelet storage period. During 5 days of storage, no dramatical change in conventional quality indicators, such as pH, were observed. To a certain extent, GSNO inhibited platelet metabolism and decreased lactate production, but more data from relevant animal experiments and clinical trials are needed to verify GSNO's function and safety in vivo. Many issues remain to be explored in the use of NO donors to improve platelet preservation quality, such as optimal concentration, time, maximum storage life, and the efficacy of adding two or more NO donors (singly or continuously). Resolution of these problems will help to promote the development of new platelet storage bags and platelet preservation solutions.

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Compliance with Ethical Standards

Conflict of interest None of the authors have any conflict of interest to declare.

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