The Alterations in Methylene Blue/Light-Treated Frozen Plasma Proteins Revealed by Proteomics

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Keywords
Fresh frozen plasma · Methylene blue/light-treated frozen plasma · Mass spectrometry · Keratin · Type II cytoskeletal 1 · Hemopexin

Abstract
Introduction: The aim of this study was to investigate the modified proteins in methylene blue/light-treated frozen plasma (MB-FP) compared with fresh frozen plasma (FFP) in order to gain a better application of MB/light-treated plasma in clinical transfusion. Methods: MB-FP and FFP were collected from Changchun central blood station, and a trichloroacetic acid/acetone precipitation method was used to remove albumin for the enrichment of lower abundance proteins. The plasma protein in MB-FP and FFP were separated using two-dimensional gel electrophoresis (2-DE) and the differentially expressed protein spots were analyzed using mass spectrometry. Finally, the differentially expressed proteins were tested using Western blot and enzyme-linked immunosorbent assay (ELISA). Results: Approximately 14 differentially expressed protein spots were detected in the MB-FP, and FFP was chosen as the control. After 2-DE comparison analysis and mass spectrometry, 8 significantly differentially expressed protein spots were identified, corresponding to 6 different proteins, including complement C1r subcomponent (C1R), inter-alpha-trypsin inhibitor heavy chain H4 (ITI-H4), keratin, type II cytoskeletal 1 (KRT1), hemopexin (HPX), fibrinogen gamma chain (FGG), and transthyretin (TTR). Western blot showed no significant difference in the expression level of KRT1 between MB-FP and FFP ($p > 0.05$). Both Western blot and ELISA indicated that the level of HPX was significantly higher in FFP than in MB-FP ($p < 0.05$). Conclusion: This comparative proteomics study revealed that some significantly modified proteins occur in MB-FP, such as C1R, ITI-H4, KRT1, HPX, FGG, and TTR. Our findings provide more theoretical data for using MB-FP in transfusion medicine. However, the relevance of the data for the transfusion of methylene blue/light-treated plasma remains unclear. The exact modification of these proteins and the effects of these modified proteins on their functions and their effects in clinical plasma infusion need to be further studied.

Introduction
Blood transfusion is a life-saving treatment for patients with severe blood loss due to various surgeries, trauma, or other clinical conditions [1]. With the continuous development of modern medicine, transfusion of whole blood, through unknown causes, may lead to circulatory overload, increased metabolic burden, and induced isoimmunity in patients, which may result in different degrees of transfusion immune response or adverse consequences in further transfusion treatment and rescue [2]. Therefore, nowadays, modern transfusion
medicine advocates blood component transfusion where doctors selectively inject different types of blood components according to the patient’s conditions [3].

Essential for blood component transfusion, plasma has been reported to play vital roles in clinical therapy (especially in intensive care and the treatment of kidney and/or hematology diseases), but it also increases the risk of transfusion-transmitted infectious diseases [4]. According to the British Society of Haematology Guidelines, fresh frozen plasma (FFP) is mainly used for the following 3 indications: prevention of bleeding (prophylaxis), hemostasis (treatment), and plasma exchange. Preventive blood transfusion is mainly performed before surgery or invasive procedures, and a large volume of trial data suggest that the use of FFP in many patients is only indicated when major bleeding occurs. For all individuals born after January 1, 1996, pathogen-inactivated plasma is recommended because the associated risk of infection with known test viruses is very low [5, 6]. The safety of blood donation has been improved through quarantine, but due to the incubation period of potential pathogens, there is still a risk for infection transmitted by blood transfusion. In any case, plasma must be safe for transfusion, especially in the situation of emerging viral pathogens that may threaten blood recipients (such as SARS-CoV-2). Several different pathogen-reduction technologies (PRT) have been adopted to reduce or prevent the spread of blood pathogens in plasma. PRT can effectively compensate for virus test defects caused by the incubation period of certain virus infections, and have been used to treat blood components and products for decades. The main processes of PRT include the use of solvent detergents, methylene blue (MB), amotosalen, and riboflavin as additives [7–10]. The most commonly used method is solvent detergents or MB. MB can bind to nucleic acids, especially G-C base pairs of the virus DNA, and MB/light treatment can result in broken DNA strands [11]. Previous studies have shown that MB can effectively inactivate viruses in plasma when exposed to light [12, 13]. Additionally, plasma after MB/light pathogen inactivation can relieve blood transfusion reaction to a great extent and is widely used in the clinic. The literature suggested that MB/light treatment can reduce the levels of several coagulation factors and their clotting ability, but still at acceptable levels [14, 15]. In addition, MB/light treatment is reported to reduce the coagulation capacity of FFP in vitro, likely due to additional freezing and thawing procedures and photooxidation-induced protein damage [16, 17]. In clinical settings, untreated FFP is preferred for patients with hemorrhage lacking clotting factors. More caution is needed when using any blood products in newborns and young children because they may develop blood transfusion complications later in life. Therefore, newborn patients are more likely to be provided with MB-treated plasma. However, in addition to blood coagulation factors, other changes in plasma protein components between MB/light-treated frozen plasma (MB-FP) and FFP still need to be explored. Through proteomics, Crettaz et al. [18] found that MB treatment resulted in alterations of fibrinogen gamma chain (FGG), transthyretin (TTR), and apolipoprotein A-1.

In order to confirm the results of Crettaz et al. [18] and discover potentially novel plasma protein changes through proteomics, this study uses two-dimensional gel electrophoresis (2-DE) to separate the plasma proteins of FFP and MB-FP, and uses mass spectrometry to analyze the spots of significantly differentially expressed protein between MB-FP and FFP. In addition to the reported changes in coagulation factors, the results of this study will also provide an additional theoretical basis for FFP or MB-FP clinical infusion for patients, and an innovative research approach for blood transfusion medicine.

Materials and Methods

Plasma Samples Collection, Processing, and Quality Control

Information

FFP samples (n = 9) and MB-FP samples (n = 9) were provided by Changchun central blood station. The MB-FP samples are identical to the commercial licensed products in China. All plasma samples are type O and RhD positive. The study was approved by the Ethics Committee of the Second Hospital of Jilin University, and written informed consent was obtained from all donors. The MB-FP samples were prepared by MB inactivation equipment with matching plasma membrane filtration, MB illumination, and MB removal systems, according to the manufacturer instructions (China Shandong Zhongbaokang Medical Equipment Co. Ltd). Briefly, 100 mL of the plasma, prepared using an integral plasma leukocyte reduction filter, was passed through the instrumentation system containing dry MB tablets and then into the illumination device. The MB concentration in the plasma was 1 ± 0.11 µM, and the plasma was exposed to 30,000–38,000 lx of visible light for 30–35 min while being agitated at about 60–80 cycles per minute at 2–8°C. Afterwards, the plasma was sent through the MB removal filter by gravity, and then was entered into a single storage container.

Resulting plasma samples contained stable clotting factors (factors V, VII, X, XI, and XIII contents ≥ 0.7 U/mL), plasma protein content ≥ 50 g/L, and MB residual quantity ≤ 0.3 µmol/L, in accordance with national standards [19]. FFP and MB-FP samples were stored at −80°C. When HBV, HCV, and HIV virus nucleic acid tests were performed on the plasma of 15,756 people, the plasma virus positive rate was 0.152% (23 HBV positive and 1 HIV positive), but no virus was detected in the plasma after MB/photochemical treatment [20]. The number of residual cells in plasma decreased by 2 orders of magnitude (from 106 to 104/unit) after MB/light virus inactivation, previously reported by Wang [21].

Extraction of Plasma Proteins

The FFP and MB-FP samples were thawed at 37°C, and then the proteins with high abundance in plasma were removed using trichloroacetic acid (TCA)/acetone precipitation methods as described previously [22, 23]. The plasma was centrifuged at 12,000 g for 5 min, and then the supernatant (20 µL) was transferred to
Fig. 1. 2-DE image comparing the proteins in MB-FP and FFP. Fourteen differentially expressed protein spots were identified between MB-FP and FFP.

The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Chicago, IL, USA) based on the manufacturer's instructions. One hundred and twenty-five micrograms of each plasma protein sample in 250 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% pH 4–7 IPG buffer, 20 mM DTT) containing 0.02% bromophenol blue was loaded to non-linear pH 4–7 IPG strips (13 cm; GE Healthcare). The IPG strips were rehydrated with protein samples for 15 h at room temperature. Isoelectric focusing (Bio-Rad Protein IEF Cell system) was performed at 20 °C with gradually increasing voltages: 100 V for 120 min, 300 V for 60 min, 1,000 V for 60 min, and then 3,500 V for 4–5 h. After IEF, the gel strips were reduced using 1% DTT in equilibration buffer (6 M urea, 50 mM Tris-HCl, 30% glycerol, 2% SDS, 0.02% bromophenol blue) for 15 min at room temperature with gentle agitation. The strips were then alkylated with 2% iodoacetamide (GE Healthcare) in equilibration buffer for the second equilibration. The balanced strips were rinsed with 1× electrophoresis buffer to remove residual solution, and then the strips were transferred to 12.5% SDS-polyacrylamide gels and sealed with 0.5% low melting agarose [24]. Electrophoresis was set at 20 °C and ran at 10 mA/gel for the first 0.15 h, followed by 20 mA/gel until the dye front reached the bottom of the gel. Finally, gels were stained with silver nitrate as previously described [25]. Triplicate 2-DE gels were performed for plasma proteins from both FFP and MB-FP samples. Gels were then scanned using ImageScanner (Shanghai Tanon Technology Co. Ltd, Shanghai, China) and differently expressed protein spots were identified by searching against a comprehensive non-redundant sequence database (NCBInr) using the MASCOT software search engine.

Western Blot
The plasma protein samples (20 µg) were separated by 12% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skimmed milk for 1 h, the membranes were incubated at 4 °C overnight with anti-Keratin, type II cytoskeletal 1 (KRT1) antibody (1:300, Wuhan Boster Biological Technology Co. Ltd, Wuhan, China), anti-hemopexin (HPX) antibody (1:300, Wuhan Boster Biological Technology Co. Ltd, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:300, Wuhan Boster Biological Technology Co. Ltd), respectively. After 3 washes with washing solution (20 mM Tris, 140 mM NaCl, and 0.1% Tween-20), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) at 37 °C for 2 h. The protein bands were visualized using the enhanced chemiluminescence detection system (Thermo, Rockford, IL, USA).

Enzyme-Linked Immunosorbent Assay
The levels of HPX in different plasma samples were determined using a Human Hemopexin/HPX ELISA (enzyme-linked immunosorbent assay) Kit (Wuhan Boster Biological Technology Co. Ltd) based on the manufacturer’s instructions. There were 3 wells for each plasma sample. Three independent experiments were performed.

Statistical Analysis
The mass spectrometry report was provided by Guangzhou FitGene Biological Engineering Co. Ltd. The results are reported as the mean ± SD. GraphPad prism 5 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses. For the comparison of 2 groups, the Student t test was used. p < 0.05 was considered statistically significant.
Results

Analysis of Differentially Expressed Proteins

The proteins of MB-FP and FFP were separated and detected by 2-DE, and the representative images of 2-DE are shown in Figure 1. After analysis, the protein matching rate of MB-FP and FFP was more than 80%, which indicated that the electrophoretic gel maps have good reproducibility and can be used to analyze differentially expressed protein spots. Compared to FFP, MB-FP had 14 differentially expressed protein spots (Fig. 1).

After processing the differentially expressed protein spots using mass spectrometry, 8 differentially expressed spots were obtained and 6 differentially expressed proteins were identified (Table 1), including complement C1r subcomponent (C1R), inter-alpha-trypsin inhibitor heavy chain H4 (ITI-H4), KRT1, HPX, FGG, and TTR. The locally magnified 2-DE maps of differentially expressed protein spots (B2, B3, B4, B5, B6, B10, C1, and C2) are displayed in Figure 2. The functions and related biological processes of the 6 differentially expressed proteins are shown in Table 2.
Verification of Differentially Expressed Proteins

The differentially expressed proteins, KRT1 and HPX, were chosen to verify their expression levels in MB-FP and FFP using Western blot and ELISA. The representative images of KRT1 and HPX expression using Western blot are shown in Figure 3a. The grey analyses showed no significant difference in the expression level of KRT1 between MB-FP and FFP ($p > 0.05$, Fig. 3b), which was not consistent with the results from 2-DE maps. For HPX, its expression was significantly higher in FFP than in MB-FP ($p < 0.05$, Fig. 3c).

ELISA was utilized to detect the concentrations of HPX in the MB-FP and FFP. The concentration of HPX in FFP was significantly higher than in MB-FP ($p < 0.05$, Fig. 4). This was in accordance with the results from Western blot. Therefore, both Western blot and ELISA results indicated the loss of HPX in MB-FP.

Fig. 2. Differentially expressed protein spots (B2, B3, B4, B5, B6, B10, C1, and C2) observed in locally magnified 2-DE maps.
In summary, MB-FP could cause changes in plasma proteins and corresponding changes in electrophoresis patterns. Modified proteins include C1R, ITI-H4, KRT1, HPX, FGG, and TTR. For the first 4 modified proteins, we are the first to discover and document their changes in MB-FP.

**Discussion**

Plasma infusion is an important part of clinical treatment. Given their long-term safety, MB/light-treated plasma is widely used in clinical practice. Compared to FFP, MB-processed plasma can reduce the incidence of adverse events in high-risk countries and reduce the spread of serious viral infections, including emerging infectious diseases transmitted through blood transfusion [12, 27, 28]. Previous studies have focused on the changes of coagulation factors, plasma protein contents, and corresponding biochemical indicators after clinical infusion of FFP and MB-FP [14–16, 28]. However, few studies have reported changes in other plasma protein components between FFP and MB-FP [18, 29, 30]. Expanding on previous research, we have discovered several novel differentially expressed proteins between FFP and MB-FP at the proteomic level through 2-DE and mass spectrometry. The 6 differentially expressed proteins identified include C1R, ITI-H4, KRT1, HPX, FGG, and TTR. The expression of these proteins in MB-FP appears to have increased. However, subsequent detection of HPX and KRT1 using ELISA and/or Western blot failed to confirm this difference. These results indicate that MB/light treatment can modify the protein components in plasma.

MB is a thiazine-based basic dye that is now widely used to inactivate virus in plasma. Crettaz et al. [18] previously reported that FGG, TTR, and apolipoprotein A-I were significantly modified in MB-FP. In addition to FGG and TTR, our results also revealed 4 additional modifications in plasma proteins, including C1R, ITI-H4, KRT1, and HPX. C1R, an important component of the complement system, participates in the complement activation pathway, as well as in inflammatory and adaptive
immune responses [31]. ITI-H4, a 120-kDa acute-phase glycoprotein, is involved in the inflammatory response of trauma. In addition, ITI-H4 has been reported to be associated with the occurrence of tumors [32]. ITI-H4 may become a potential therapeutic target that could inhibit cancer metastasis, as well as a prognostic marker for patients [33]. FGG can form an insoluble fibrin matrix when combined with alpha chain and beta chain, which plays a role in blood clot and fibrin clot formation, as well as hemostasis [34]. Cardigan et al. [35] previously reported that MB treatment resulted in a 10% reduction in endogenous thrombin potential and 30% decrease in peak thrombin, as well as an expected 20–35% loss of Factor (F)VIII, fibrinogen, and FXI activity, but MB treatment has very little effect on the rate and the strength of clot formation as assessed using thromboelastometry. The FGG modifications we observed may explain or be related to the above results of Cardigan et al. [35]. TTR is not only a plasma protein, but also a carrier protein that can transport thyroid hormones and retinols [36]. KRT1, an essential component of the cytoskeleton, can activate carbohydrate binding receptor and participate in the lectin pathway [37]. Western blot results showed that KRT1 was determined both in MB-FP and FFP with no significant difference, but it was detected only in MB-FP using 2-DE maps. A possible explanation is that the level of KRT1 in plasma is low, which is due to low levels of plasma particles and residual cells in plasma. Another explanation may be that Western blot detection is more sensitive than 2-DE and amplifies protein signals. HPX is associated with the above results of Cardigan et al. [35]. TTR is not only a plasma protein, but also a carrier protein that can transport thyroid hormones and retinols [36]. KRT1, an essential component of the cytoskeleton, can activate carbohydrate binding receptor and participate in the lectin pathway [37]. Western blot results showed that KRT1 was determined both in MB-FP and FFP with no significant difference, but it was detected only in MB-FP using 2-DE maps. A possible explanation is that the level of KRT1 in plasma is low, which is due to low levels of plasma particles and residual cells in plasma. Another explanation may be that Western blot detection is more sensitive than 2-DE and amplifies protein signals. HPX is mainly produced in the liver, and partially expressed in the central and peripheral nervous system, skeletal muscle, retina, and kidney [38]. HPX can promote the metabolism of heme and iron, thus protecting the body from oxidative damage [39]. In our research, the HPX level was significantly lower in MB-FP than in FFP, as revealed by both Western blot and ELISA. These results suggest that MB/light treatment caused a decrease in HPX. But why was the decrease in HPX inconsistent with the high level of HPX shown using 2-DE maps? Despite the overall low level of HPX in MB-FP, we speculate that the high level of HPX shown using 2-DE maps accounted for one of the HPX isoforms, which may have been modified by MB/light treatment. Therefore, these protein modifications in MB-FP caused by MB/light treatment may have important clinical implications.

Evidenced by many publications on its quality and application in current clinical use, MB/light-treated plasma is considered to be highly safe and widely used. However, current knowledge is limited regarding what kinds of proteins in plasma can be changed by the process of MB/light treatment while inactivating plasma viruses. On the other hand, the changes in plasma protein caused by MB/light treatment can promote researchers to improve the method of MB/light treatment of plasma, such as adding other viral inhibitors to reduce the amount of MB. On the other hand, we can further study whether the plasma protein changes are related to the specific disease of the plasma transfusion patient and adjust the treatment plan accordingly.

In conclusion, this comparative proteomics study revealed that MB/light treatment modifies plasma proteins, such as C1R, ITI-H4, KRT1, HPX, FGG, and TTR. Future research may benefit from studying the exact modification of these proteins and the effects of these modified proteins on their functions and utilities in clinical plasma infusion.

Statement of Ethics
The experiment was approved by the Ethics Committee of the Second Hospital of Jilin University, and written informed consent was obtained from all donors.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

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Author Contributions
T.W. and J.L. designed the research study. T.W., X.W., and K.R. conducted the experiment and obtained the data. K.R. and X.H. analyzed and interpreted the results. T.W. drafted the manuscript, and J.L. provided revisions. All authors read and approved the final version.

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