The effect of methylene blue photoinactivation and methylene blue removal on the quality of fresh-frozen plasma

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BACKGROUND: The effects of using fresh or frozen-thawed plasma, WBC reduction of plasma before freezing, and the use of two different methylene blue (MB) removal filters on the quality of MB-treated plasma were compared.

STUDY DESIGN AND METHODS: In a paired study (n = 11/arm) plasma was frozen within 8 hours of collection, thawed, MB photoinactivated, and then filtered using one of two MB removal filters. Fresh plasma (n = 16) and plasma WBC reduced before freezing (n = 19) were MB inactivated.

RESULTS: Freeze-thawing resulted in loss of activity of FXII and VWF of 0.06 and 0.04 units per mL, respectively, but no significant loss of activity of factors II through XI or fibrinogen. Further loss of activity occurred after MB treatment: FII (0.07 IU/mL), FV (0.11 U/mL), FVII (0.08 IU/mL), FVIII (0.28 IU/mL), F IX (0.12 IU/mL), FX (0.16 IU/mL), FXI (0.28 U/mL), FXII (0.15 U/mL), VWF antigen (0.05 IU/mL), VWF activity (0.06 U/mL), and fibrinogen (0.79 g/L). Losses due to this step were significantly (5-10%) lower in fresh plasma compared to frozen-thawed plasma. Neither MB removal filter resulted in significant loss of activity of any factor studied.

CONCLUSION: MB removal, by either of the available filters, has little impact on the coagulation factor content of plasma, but freezing of plasma before MB treatment results in a small additional loss.

Due to stringent donor selection and testing procedures, fresh-frozen plasma (FFP) in the developed world offers a high degree of viral safety. For example, the risk of an infectious FFP donation entering the blood supply in England is estimated to be 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, 2002). Nevertheless, viral transmission from blood components continues to occur, with 16 cases reported in the UK in the last 6 years.[1] There is, therefore, considerable research activity in pathogen inactivation of single-unit components because methods suitable for single components offer reassurance that no increased infectious risks are added due to pooling. For FFP only, one licensed single-unit system is currently available (methylene blue photoinactivation). It is desirable that there is as much flexibility as possible in the handling conditions for plasma before inactivation, to enable production of FFP from collection centers distant from the processing site. This is particularly relevant because the UK Departments of Health have recently recommended that FFP is imported from North America for neonates and children born after 1995 (after the introduction of relevant food bans to limit BSE transmission) as a precautionary measure against vCJD transmission. Previous studies have

ABBREVIATIONS: APC = allophycocyanin-conjugated; APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; PMN, = neutrophil; PRP = platelet-rich plasma; PT = prothrombin time; VWF:CB = VWF collagen-binding activity.

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demonstrated vCJD infectivity in plasma of rodents infected with prion diseases,[2,3] and a recent report describes interim results from a study that demonstrate transmissions of bovine spongiform encephalopathy (BSE) and scrapie between sheep by whole-blood transfusion.[4] Because background levels of virus marker positivity in the North American population are significantly higher than in the UK, it has been deemed sensible to subject imported plasma to a pathogen-inactivation step.

The methylene blue (MB) photoinactivation process for viral inactivation of human plasma has been well described[5] as has its effect on the loss of coagulation factor activity of plasma.[5-9] The original Springe MB process, also used by Grifols in Spain, described by Lambrecht,[5] used freeze-thawing of plasma before MB inactivation to expose intracellular viruses to the action of MB. However, recently, blood collection packs that integrate WBC reduction and MB addition before inactivation of plasma (Baxter Pathinact, Baxter Healthcare, Compton Newbury, Berkshire, UK, and Maco Pharma Theraflex, Middlesex, UK) remove the need to freeze-thaw plasma.[10-12] There are also differences between the systems in how MB is added to plasma. With two of the systems (Springe and Baxter), a variable dose of MB solution is added to achieve a standard final concentration of 1 μ*M* MB. The other system (Maco Pharma Theraflex) incorporates an 85-μg pellet of MB hydrochloride per plasma unit, therefore the concentration can vary slightly (0.84-1.13 μ*M*) depending upon the plasma volume (recommended range, 235-315 mL).

For MB photoinactivation of plasma to be centralized, but plasma from remote sites used as a start material, it is essential to be able to freeze and thaw plasma before treatment. Although we have previously evaluated the use of the two systems (Baxter and Maco Pharma) using fresh plasma,[10-12] we have not evaluated freeze-thawing of plasma before MB treatment using such systems. Although it is known that freeze-thawing itself has minimal effect on the coagulation factor activity of plasma,[7] there are no comparative data available on whether the loss of coagulation factor activity due to the MB inactivation step is affected by prior freeze-thawing. Furthermore, in the UK, there was concern that freeze-thawing non-WBC-reduced plasma could potentially increase exposure to vCJD due to fragmentation of platelets and WBCs, which are known to contain normal cellular prion protein (Prp^c)[13] and might therefore host the infective abnormal prion protein Prp^{sc}. We therefore assessed the effect of removing these cells by an additional WBC reduction step before freezing on the quality of MB plasma.

Following concerns over possible side effects of residual MB in plasma, a further recent development is the ability to remove MB by filtration before final component storage. Evaluations of two removal filters (Pall MB1, Pall Biomedical, Portsmouth, UK, and HemaSure LeukoVir, Marlborough, MA) MB have been previously reported,[14,15] but there are no data available on the use of a new MB removal filter (Maco Pharma Blueflex). The aim of this study was therefore to evaluate the combined effect of WBC reduction before freezing, freeze-thawing, MB photoinactivation, and MB removal using two different filters, on coagulation factor activity and activation markers in FFP. We also examined the effect of freeze-thawing and subsequent filtration of non-WBC-reduced plasma on its cellular constituents, to provide assurance that the process is not likely to increase the risk of vCJD transmission after transfusion to patients.

MATERIALS AND METHODS

Blood collection and processing

The experimental design is shown in Fig. 1. Twenty-four units of whole blood (group A, n = 12; group O, n = 12) were collected into "Top and Bottom" configuration blood packs (Pall Medsep 789-94 U, Pall Biomedical). Blood was then centrifuged (Heraeus Cryofuge 6000, Kleinostheim, Germany) at 3300 rpm for 12 minutes at 22°C and processed to RBCs and plasma (Compomat G4 system, Fresenius-Hemocare NPBI, Abingdon, UK). In Experiment A, plasmas were pooled in groups of two units of identical ABO group into 600-mL transfer packs (Baxter FGR2089, Baxter Healthcare). The pools were mixed thoroughly and divided equally between two 300-mL transfer packs (Fresenius Hemocare P4164, Fresenius Hemocare). All units of plasma were frozen within 8 hours of collection in a freezer (Thermogenesis MP1101, Cheshire, UK) to −45°C within 45 minutes and stored frozen at −40°C for 4 days to 4 weeks. The units were then thawed at 37°C and immediately WBC reduced (PLAS 4, Maco Pharma, Middlesex, UK) and MB photoinactivated (Maco Pharma Maco-Tronic system) as previously described.[11] For each pair of plasmas, MB was removed (either Pall MB1 or Maco Pharma Blueflex) according to the manufacturers' instructions. Plasmas were refrozen in a freezer (Thermogenesis MP1101) and stored at −40°C. In addition, 19 units of plasma were WBC reduced before freezing using one of two filters (RZ2000, Baxter Healthcare, or LPS1, Pall Biomedical), MB inactivated, and then MB removed (MB1 filter, Experiment B). These filters were selected because they are known to have minimal effect on coagulation factors in plasma.[16] A further 16 units of plasma (group O, n = 8; group A, n = 8) were MB inactivated without the freeze-thaw step and MB removed (Blueflex filter, Experiment C).

We took 15-mL samples by sterile connection of a sample pouch at four time points: 1) before freezing, 2) after thawing and before WBC reduction and MB addition, 3) after MB treatment before MB removal, and 4) after MB removal. Samples were frozen at −80°C for coagulation assays, and two aliquots were frozen in EDTA for C3a des arg assays.

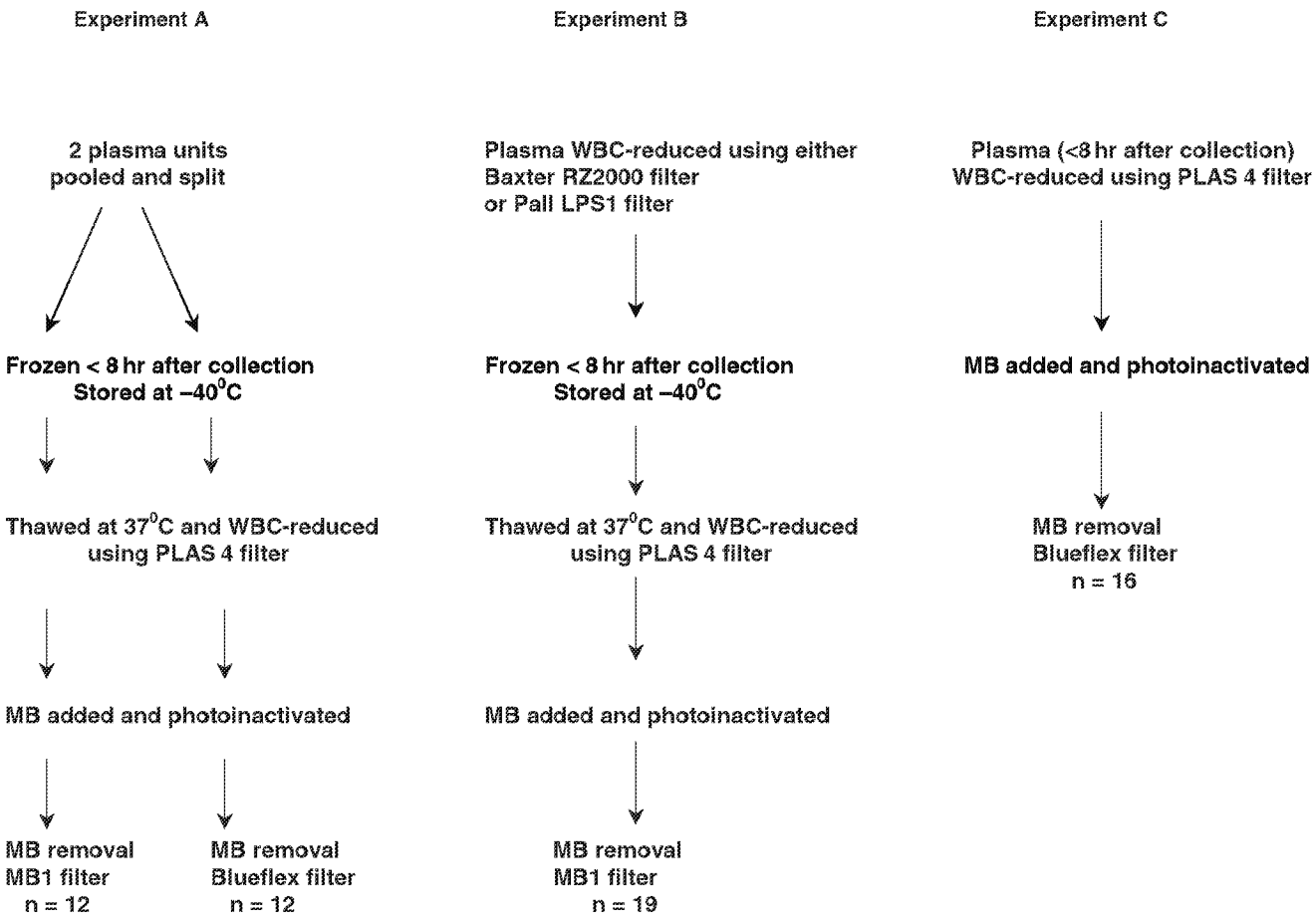

Fig. 1. Study design.

Plasma factors

All coagulation assays were performed using commercially available analyzers (Sysmex CA 1500 analyzer, Sysmex, Milton Keynes, UK; Coagamate X2 analyzer, Organon-Teknika, Cambridge, UK; or Amelung KC 4 A micro analyzer, Sigma Diagnostics, Poole, Dorset, UK). FII, FV, FVII, and FX were assayed by one-stage prothrombin time (PT)-based assays and F IX and FXII using a one-stage activated partial thromboplastin time (APTT)-based assay, using deficient plasma (Dade Behring, Marburg, Germany). The PT and APTT were expressed as a ratio to the geometric mean result of 20 normal citrated plasmas. These types of samples were chosen as "normal" plasma to provide a standard reference point between studies. VWF antigen was measured by latex agglutination (STA Liatest Kit, Diagnostica Stago, Asnieres, France). FVIII and FXI were assayed using one-stage clotting assays with deficient plasma (Diagnostics Scotland, Edinburgh, Scotland; and Sigma-Aldrich Company, Poole, Dorset, UK, respectively). Fibrinogen was measured using a Clauss assay with Fibriquick reagents (Organon-Technika, Cambridge, UK). FVIII assays were standardized using the British plasma standard (NIBSC, South Mimms, UK). All other assays were standardized using Coagulation Reference plasma 100 percent (Technoclone, Dorking, UK). A control plasma of known potency was assayed on each occasion for all coagulation assays.

Commercially available ELISA kits were used to determine levels of prothrombin fragment 1 + 2 (Dade-Behring), FXIIa (Axis-Shield, Dundee, Scotland), and VWF collagen-binding activity (VWF:CB, Immuno, Vienna, Austria). C3a des arg was assayed by radioimmunoassay (Amersham Pharmacia Biotech, Buckshire, UK). VWF cleaving protease activity was measured as previously described[17] and results expressed as a ratio to that of a pooled normal citrated plasma.

Effect of freeze-thawing plasma and filtration steps on cellular content of plasma

Double WBC-reduced plasma (LPS1 filter, Pall Biomedical) was spiked with WBCs (<1-200 $\times 10^6$/L) with or without platelets (<1-100 $\times 10^9$/L), both of which were prepared from fresh whole blood by density gradient centrifugation,

to represent levels of cellular contamination that may be expected to occur in non-WBC-reduced plasma. Plasma was then blast-frozen, thawed, WBC-reduced by sterile connection with the (PLAS 4) filter, MB added, and MB removed using a filter (the Pall MB1 filter). Samples were taken at four stages: before freezing, after thawing, after PLAS4 LD filter, and after MB removal. Samples were analyzed for platelet count by a hematology analyzer (Sysmex SE9000, Sysmex) and WBC count by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) using LeucoCount reagents (Becton Dickinson). Release of the neutrophil primary granule marker elastase was measured by ELISA of α_1-proteinase inhibitor: neutrophil (PMN) elastase complexes (Pathway Diagnostics, UK) and release of LDH by enyzymatic assay in supernatant plasma (Vitros DT60II, Axis-Shield, Dundee, Scotland). RBC microparticles were measured by flow cytometry (FACSCalibur) as previously described using antibodies to glycophorin A.[18]

Analysis of platelet microparticles (PMP) was determined as follows: Plasma (5 µL) was incubated for 20 minutes at room temperature with 5 µL allophycocyanin-conjugated anti-CD61 (APC-CD61, Caltag-Medsystems, Towcester, UK), 5 µL rhodophycoerythrin-conjugated anti-CD42b (Caltag-Medsystems), 10 µL FITC annexin V (FITC-AV, Caltag-Medsystems), 5 µL 10 × HBSS (Sigma, Poole, UK) and made up to 50 µL with HEPES-calcium buffer (2.8 m*M* $CaCl_2$, 20 m*M* HEPES). Samples were resuspended in 0.45 mL of 1 × HBSS and transferred to a tube containing a known amount of beads (Trucount, Becton Dickinson), and analyzed using a flow cytometer (FacsCalibur, Becton-Dickinson). Platelet microparticles were defined using forward scatter as events falling in a region, which includes less than 2 percent of platelets in plasma (PRP) from 20 normal donors, and of less than 1 µm as determined by APC fluorescent beads (Spherotec, Libertyville, IL). Platelet-derived events were defined by fluorescence due to APC-CD61 binding above that of an isotype-matched control. Annexin-V-positive events were defined as events binding FITC-AV above a control containing 5 m*M* Na_3 EDTA. In normal subjects (n = 20), less than 1 percent of unstimulated platelets bind FITC-AV. To control assay variability, a negative control of unstimulated PRP and a positive control (PRP incubated with 10 µ*M* A23187 [Calibochem-Novabiochem, San Diego, CA] for 15 min) were included for platelet microparticle assays.

Effect of MB on assays

To assess the effect of the presence of MB itself on coagulation factor assays, a MB pellet (from the Maco Pharma pack) was dissolved in each of six units of plasma. Samples were collected before and after addition of MB, and once MB had been added, the plasma was not photoilluminated and was protected from light at all times. All parameters were performed as for the main study with the exception that all coagulation assays were performed using a particular analyzer (Sysmex CA 1500 analyzer) and C3a des arg levels were performed by ELISA (Quidel, San Diego, CA).

Statistical analysis

Since the distribution of some data were non-Gaussian with positive skew, nonparametric tests were applied. The Wilcoxon rank sum test was used for paired data and the Mann-Whitney U-test for unpaired data. A p value less than 0.05 was considered significant. All results are given as median with range.

RESULTS

Plasma processing

From the 24 paired units (Experiment A), one unit of plasma fractured on thawing, therefore data on 11 paired units of plasma are presented, all of which were within the required volume range before MB inactivation. Due to sampling, 2 out of 16 fresh plasma units (Experiment C) were slightly below the lower range limit (233 and 234 mL). Filtration time for one WBC-reduction filter (PLAS 4) was 8 to 15 minutes, with a loss of 20 mL of plasma. Filtration times for two other removal filters (Pall MB1 and Maco Blueflex) were 2 to 5 minutes and 5 to 11 minutes, respectively, with a loss of 10 mL of plasma for each.

Effect of freeze-thawing and MB on loss of coagulation factors

The change in coagulation activity due to freeze-thawing and the MB process is shown in Table 1. There was a significant loss of FXII (2%) and VWF:CB (9%), and a small increase in levels of FVII (1%) and FXI (4%) due to freeze-thawing. This was associated with an increase in both PT ratio (1.06 [0.97-1.12] vs. 1.05 [0.95-1.11], $p < 0.0001$) and APTT ratio (1.04 [0.91-1.20] vs. 1.02 [0.89-1.18], $p < 0.0001$). The degree of loss of coagulation activity due to the MB process varied between factors, the highest losses occurring with FVIII (29%), fibrinogen (28%), and FXI (25%), therefore the evaluation of plasma treated fresh was mainly restricted to these factors. For FV, FVIII, FXI, and fibrinogen, the loss of activity due to the MB process was approximately 8-percent higher in the frozen-thawed plasma units compared with fresh plasma (Table 1). In addition, the increase due to this step in both PT ratio (0.09 [0.04-0.16] fresh vs. 0.14 [0.08-0.28] frozen-thawed, $p < 0.0001$) and APTT ratio (0.11 [0.05-0.15] fresh vs. 0.16 [0.11-0.26] frozen-thawed, $p < 0.0001$) was also higher. However, there was no significant difference in changes in levels of FVII, FXIIa, and C3a due to MB treatment between units which were treated fresh or after freeze-

TABLE 1. Percentage change in coagulation factor activity due to freeze-thawing and MB treatment of plasma

Factor	Due to freeze-thawing median (range)	Due to MB treatment + WBC reduction* (freeze-thawed plasma) median (range)	Due to MB treatment + WBC reduction (fresh plasma) median (range)
Number	22	22	16
Fibrinogen (g/L)	–10 (–27 to 14)	–28 (–51 to –20)	–21 (–38 to –7)†
FII (IU/mL)	0 (–4 to 4)	–8 (–11 to –2)	NA
FV (U/mL)	0 (–5 to 4)	–13 (–20 to 4)	–5 (–11 to 4)†
FVII (IU/mL)	1 (–1 to 4)‖	–7 (–10 to –1)	–4 (–9 to –1)
FVIII (IU/mL)	–3 (–20 to 9)	–29 (–42 to –9)	–24 (–37 to –11)†
F IX (IU/mL)	1 (–3 to 4)§	–13 (–20 to –11)	NA
FX (IU/mL)	0 (–2 to 3)	–15 (–22 to –10)	NA
FXI (U/mL)	4 (–19 to 11)‖	–25 (–35 to –7)	–15 (–23 to –6)†
FXII (U/mL)	–2 (–6 to 1)‖§	–18 (–31 to –14)	NA
VWF:Ag (IU/mL)	–1 (–4 to 3)§	–6 (–11 to –3)	NA
VWF:CB (U/mL)	–9 (–17 to 5)‖§	–8 (–16 to 3)	NA
FXIIa (ng/mL)	0 (–25 to 43)	–20 (–43 to 33)	–14 (–43 to 0)
Prothrombin *F*1 + 2 (n*M*)	–19 (–55 to 32)¶	91 (36 to 160)	27 (–12 to 180)‡
C3a des arg (ng/mL)	0 (–41 to 65)	–10 (–45 to 155)	16 (–27 to 295)

* WBC reduction was performed with an integral PLAS 4 WBC-reduction filter in the Maco Pharma MB pack configuration.
† $p < 0.01$ refers to significance from the Mann-Whitney U-test between fresh and frozen plasma.
‡ $p < 0.05$.
§ $n = 11$.
¶ $p < 0.01$ refers to significance from the Wilcoxon rank sum test between plasma before freezing and after thawing.
‖ $p < 0.05$.

thaw. The increase in prothrombin *F*1 + 2 levels due to MB treatment was higher in frozen-thawed units compared to fresh.

When the influence of MB on the assays was studied (in the absence of photoinactivation), there was no significant difference between before or after the addition of MB for any parameters, apart from FXIIa, which was significantly lower after MB addition (1.59 [0.76-2.13] ng/mL before, 0.72 [0.56-1.05] ng/mL after, $p < 0.05$ before vs. after).

Effect of MB removal filters on coagulation activity

To assess the difference between the two MB removal filters, pairs of units were pooled and half of each pool MB-treated and processed through each of the removal filters in parallel. Due to logistical problems, it was not possible to process and assay these two sets simultaneously. A small difference was apparent in levels of some coagulation factors between the two arms of the study, probably due to small differences in processing and storage. However, the percentage change in activity due to freeze-thawing and MB treatment was the same for each arm of the study (data not shown), and therefore these data were combined. To evaluate the effect of MB removal filters, a comparison of pre- and postcoagulation activity for each arm of the study was examined (Table 2). There was no apparent decrease in any parameter studied with either removal filter, apart from a reduction in levels of C3a using one of the filters (Pall MB1). There was a slight increase in levels of fibrinogen, FII, FV, FVII, and FX, using the Pall filter. There was an apparent increase in levels of FXIIa after filtration with both removal filters, which was probably due to the influence of MB on the assay and was comparable between filters. There was an extremely small variation in the PT and APTT ratios with both filters (Table 2).

We compared the final levels of coagulation activity in frozen-thawed MB-treated plasma to a reference range based on 66 samples of WBC-reduced plasma that had not been MB treated. Because there was no loss of activity with either MB removal filter, both sets of data were pooled. The reference data was not collected as part of this study but from previous studies carried out by the National Blood Service over the past 4 years. The methodology used in these studies[16] for either plasma processing or assay did not differ significantly from the current study. Over 90 percent of MB units were within our reference range for all coagulation factors, apart from prothrombin *F*1 + 2 and PT ratio, the MB-treated plasma having 23 percent and 50 percent of values above the range, respectively (Table 3). The range of PT ratios observed in reference and MB-treated plasma is shown in Fig. 2.

We did not evaluate MB removal by the filters used in this study, but previous studies have shown that the MB1 filter removes 81 to 95 percent of MB[14] and the Blueflex filter removes more than 95 percent MB.[19]

Effect of WBC reduction before freezing

WBC reduction of plasma before freezing appeared to have little influence on final levels of FVIII (0.67 [0.44-1.23] WBC reduced vs. 0.62 [0.48-0.86 IU/mL] non-WBC reduced) or fibrinogen (1.88 [1.45-3.24] WBC reduced vs.

TABLE 2. Effect of MB-removal filters on plasma coagulation activity using frozen-thawed MB photoinactivated plasma

	MB1 filter median (range)		Blueflex filter median (range)	
Factor	Before MB removal	After MB removal	Before MB removal	After MB removal
Number	11		11	
PT (ratio)	1.17 (1.11-1.34)	1.07 (1.03-1.24)*	1.23 (1.13-1.33)	1.22 (1.13-1.32)*
APTT (ratio)	1.11 (1.05-1.28)	1.13 (1.06-1.30)†	1.25 (1.17-1.39)	1.27 (1.19-1.43)*
Fibrinogen (g/L)	1.88 (1.30-2.13)	1.93 (1.28-2.27)*	2.04 (1.37-2.13)	1.96 (1.28-2.32)
FII (IU/mL)	0.96 (0.77-1.04)	1.00 (0.78-1.04)†	0.95 (0.77-1.04)	0.97 (0.77-1.04)
FV (U/mL)	0.78 (0.55-0.86)	0.80 (0.56-0.91)*	0.76 (0.58-0.97)	0.76 (0.58-0.88)
FVII (IU/mL)	0.99 (0.79-1.43)	1.02 (0.83-1.55)*	1.01 (0.77-1.40)	1.00 (0.78-1.45)
FVIII (IU/mL)	0.63 (0.47-0.89)	0.62 (0.48-0.86)	0.61 (0.48-0.79)	0.61 (0.46-0.76)
F IX (IU/mL)	0.96 (0.78-1.06)	0.96 (0.83-1.11)		
FX (IU/mL)	0.95 (0.70-1.12)	1.02 (0.75-1.15)*	0.95 (0.72-1.09)	0.96 (0.73-1.07)
FXI (U/mL)	0.77 (0.57-0.99)	0.77 (0.59-0.99)	0.75 (0.55-0.99)	0.71 (0.58-0.78)
FXII (U/mL)	0.88 (0.41-1.07)	0.88 (0.40-1.09)		
VWF:Ag (IU/mL)	0.99 (0.74-1.19)	0.98 (0.75-1.18)	0.92 (0.70-1.09)	0.93 (0.70-1.09)
VWF:CB (U/mL)	0.60 (0.47-0.68)	0.73 (0.42-0.85)	0.70 (0.59-0.87)	0.74 (0.56-0.82)
FXIIa (ng/mL)	1.50 (0.40-2.50)	1.80 (0.70-2.90)*	1.25 (0.75-2.00)	1.50 (1.00-2.25)*
Prothrombin *F*1 + 2 (nM)	0.96 (0.61-1.75)	0.88 (0.76-1.83)	0.74 (0.58-1.19)	0.75 (0.52-1.25)
C3a des arg (ng/mL)	438 (207-1928)	304 (107-431)*	337 (225-1909)	302 (226-1713)

* $p < 0.01$ refers to statistical significance from the Wilcoxon rank sum test between plasma before and after MB removal.
† $p < 0.05$.

TABLE 3. Final levels of coagulation factors and activation markers in freeze-thawed MB-photoinactivated plasma after MB removal

Factor	Final level in plasma*	Reference range†	Units in range (%)
Number	22	66	
PT (ratio)	1.16 (1.03-1.32)	1.05 (0.95-1.16)‡	50
APTT (ratio)	1.24 (1.06-1.43)	1.09 (0.86-1.36)‡	95
Fibrinogen (g/L)	1.95 (1.28-2.32)	1.10-4.30	100
FII (IU/mL)	0.98 (0.77-1.04)	0.70-1.20	100
FV (U/mL)	0.79 (0.56-0.91)	0.50-1.40	100
FVII (IU/mL)	1.01 (0.78-1.55)	0.60-1.40	100
FVIII (IU/mL)	0.62 (0.46-0.86)	0.40-1.60	100
F IX (IU/mL)	0.96 (0.83-1.11)§	0.60-1.40	100
FX (IU/mL)	1.01 (0.73-1.15)	0.70-1.30	100
FXI (U/mL)	0.75 (0.58-0.99)	0.60-1.30	91
FXII (U/mL)	0.88 (0.40-1.09)§	0.40-1.50	100
VWF:Ag (IU/mL)	0.96 (0.70-1.18)	0.60-1.65	100
VWF:CB (U/mL)	0.74 (0.42-0.85)	0.50-1.50	91
FXIIa (ng/mL)	1.50 (0.40-2.90)	0.50-5.00	100
Prothrombin *F*1 + 2 (n*M*)	0.85 (0.52-1.83)	0.20-1.10	77
C3a des arg (ng/mL)	303 (107-1713)	1117 (0-12,330)	100

* Data (n = 22) are represented by the median (range) from plasma MB-removed by MB1 filter (n = 11) and Blueflex filter (n = 11).
† Reference range of normal plasmas is defined as the mean ± 2 SD for normally distributed data and the geometric mean with 95-percent CI for skewed data based on WBC-reduced FFP. Percentage of units in range is defined as above the lower limit for coagulation factors and below the upper limit for PT, APTT, and activation markers.
‡ n = 100.
§ n = 11, using MB1 filter only.

1.70 [1.24-2.31 g/L] non-WBC reduced) in plasma subsequently MB treated and removed using the MB1 filter. VWF cleaving protease (VWF:CP) activity was measured in four MB-inactivated plasmas, which were WBC reduced before freezing and MB-removed using the MB1 removal filter. VWF:CP results ranged from 0.81 to 1.00 (normal range, 0.80-1.20 in citrated plasma). We have not assessed VWF:CP activity in plasma MB-depleted using the Blueflex filter. Quality-monitoring data from routinely processed units (n = 225), WBC-reduced before freezing, MB-treated, and MB-removed using the MB1 filters, showed a mean volume of 242 mL (SD = 19) and FVIII content of 0.79 IU per mL (SD = 0.25). These results comply with UK specifications for MB-treated FFP.[20]

Effect on cellular content of plasma

When platelets were spiked into plasma, there was no consistent difference between levels measured before and after freeze-thawing when measured by hematology analyzer, but levels were consistently lower after thawing when measured by flow cytometry (Table 4). When WBCs were spiked into plasma, there appeared to be a trend for lower WBC counts and higher levels of α_1-proteinase inhibitor: PMN elastase complexes in plasma after freezing (Table 4). However, in the absence of platelets, levels of LDH did not increase substantially. Freeze-thawing of plasma resulted in an increase in levels of platelet microparticles as well as microparticles characterized by the binding of purified annexin V (Table 4). Platelet microparticles were reduced to levels observed in fresh WBC-reduced plasma or below after WBC reduction of frozen plasma with the PLAS 4 filter. However, a significant proportion (~30%) of microparticles characterized by annexin V binding were not removed by the WBC-reduction or the MB1 removal step. These also appeared to be derived solely from plate-

lets because levels after WBC reduction in samples spiked with WBCs in the absence of platelets were not different from WBC-reduced plasma alone. However, even at an added platelet count of 30×10^9 per L (Sample D, the current UK specification), the number of annexin V-positive microparticles in frozen-thawed plasma subsequently filtered using the PLAS 4 filter (43×10^9/L) is similar to that seen in our current routine WBC-reduced non-MB-treated plasma product (mean residual platelet count of 3 $\times 10^9$/L).

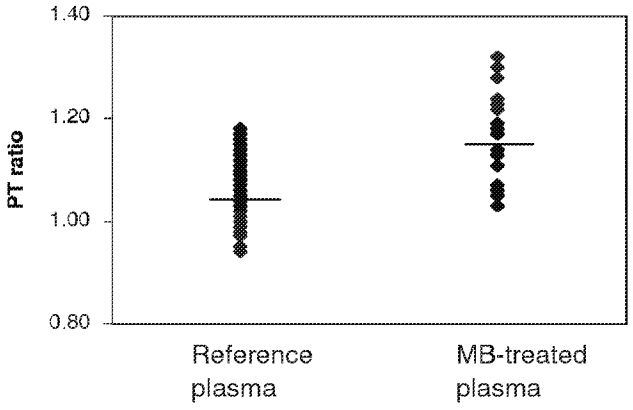

Fig. 2. PT ratio in MB-treated (n = 22) or reference plasma (n = 100). For MB-treated plasma, MB was removed by MB1 filter (n = 11) and Blueflex filter (n = 11). Reference plasma is historical data from WBC-reduced FFP. Horizontal bar represents the median value. The PT is expressed as a ratio to the geometric mean result of 20 normal citrated plasmas.

Before freezing, levels of RBC microparticles were 8 (5-12 $\times 10^9$/L), increasing by 33 percent after freeze-thawing. This was not related to platelet or WBC content, and levels after WBC reduction were below the detection of the assay system used (data not shown).

DISCUSSION

MB treatment of plasma has been shown to inactivate 4 to 6 logs of transfusion-transmitted viruses, including HIV, HBV, parvovirus B19, and West Nile virus.[5,21,22] Original studies on MB inactivation were reported on plasma freeze-thawed before treatment.[5,7] Later, work on other systems (Baxter Pathinact and Maco Pharma Theraflex systems) was performed on fresh plasma.[10-12] However, there are no data available on the difference between using fresh or freeze-thawed plasma as a starting component for MB treatment. In our study, freeze-thawing of plasma resulted in a small loss of FXII and VWF:CB activity

TABLE 4. The effect of freeze-thawing, WBC reduction, and MB removal on cellular components of plasma

	Spike*									
	A	B	C	D	E	F	G	H	I	J
Platelets – HA† (10^9/L)										
Before freeze	<3	<3	10	31	108	<3	<3	<3	<3	<3
After thaw	<3	6	10	32	97	<3	<3	<3	<3	<3
Platelets – FC‡ (10^9/L)										
Before freeze	<0.5	5.9	10.8	32.0	125.1	<0.5	<0.5	<0.5	<0.5	<0.5
After thaw	<0.5	4.8	9.0	24.6	92.2	<0.5	<0.5	<0.5	<0.5	<0.5
WBCs (10^6/L)										
Before freeze	<1	8.9	29.6	90.7	233.8	<1	1.8	14.1	50.6	92.9
After thaw	<1	9.1	28.1	94.0	210.9	<1	1.6	13.2	43.4	89.5
PMN elastase (µg/L)										
Before freeze	46.4	39.7	37.5	46.3	57.0	20.6	23.7	24.0	25.3	25.7
After thaw	37.5	35.8	42.2	53.5	79.8	22.8	23.1	30.4	43.2	58.8
After WBC reduction	34.5	35.3	40.8	57.4	77.4	21.1	20.5	27.0	41.2	53.5
After MB1 filter	35.1	37.0	45.1	58.2	71.9	18.1	21.3	24.8	37.9	57.9
Platelet microparticles (10^9/L)										
Before freeze	0.2	0.2	0.4	0.5	1.0	0.3	0.5	0.4	0.3	0.9
After thaw	0.1	0.5	0.7	2.6	10.1	0.1	0.1	0.0	0.3	0.0
After WBC reduction	0.0	0	0	0.1	0.2	0	0	0	0.1	0
After MB1 filter	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.0	0.3
Annexin V +ve microparticles (10^9/L)										
Before freeze	6	7	8	10	13	4	4	4	5	4
After thaw	12	31	54	138	574	9	9	9	13	13
After WBC reduction	3	10	20	46	198	5	2	3	5	5
After MB1 filter	3	8	15	43	196	4	4	4	4	8
LDH (U/mL)										
Before freeze	393	400	423	476	732	390	381	388	376	382
After thaw	384	404	434	523	796	388	386	385	393	395

* WBC-reduced plasma units were spiked with WBCs alone (Samples G-J) or WBCs and platelets (Samples B-E) to the concentrations shown in the before freeze rows. Samples A and F were not spiked. Plasma was frozen-thawed, WBC reduced using the PLAS 4 filer; MB added and MB removed using the MB1 filter. Results are from a single experiment. Platelets and WBCs were not detectable following WBC reduction.
† FC-flow cytometry.
‡ HA-haematology analyser.

as well as a minor prolongation of PT and APTT. Interestingly, there was an apparent increase in activity of FVII and FXI on freeze-thawing, presumably reflecting small changes in the activation status of these factors. However, other factors studied remained unchanged. This agrees with the work of Zeiler et al.,[7] who showed that the loss of coagulation activity in MB-treated plasma was mainly attributable to the MB photoinactivation step rather than freeze-thawing of plasma. The loss of activity observed in frozen-thawed units due to MB photoinactivation in our study was similar to that previously reported.[5,7] For the variables we studied, with the exception of FVII, loss of coagulation factor activity due to the MB-inactivation step (including the WBC reduction filter) was 8-percent higher when frozen-thawed plasma units were used rather than fresh. In addition, the increase in prothrombin *F*1 + 2 levels after MB inactivation and WBC reduction was higher in units frozen-thawed compared with fresh plasma, indicating a higher degree of thrombin generation. This was not associated with an increase in FXIIa. Because our MB process includes an integral WBC-reduction step, we cannot determine whether the differences seen between fresh and frozen-thawed plasma are attributable to the WBC-reduction or MB process. The WBC-reduction filter used in the MB packs has previously been shown to have minimal effect on coagulation activity (unpublished data) using fresh plasma, but this could be different for frozen-thawed plasma.

We also sought to compare the effect of two different types of MB removal filters (Maco Pharma Blueflex or Pall MB1 filter) on plasma factor activity. Neither filter resulted in loss of any variable studied. Both MB removal filters resulted in a small increase in the APTT ratio, which might be attributable to contact activation of plasma with the filter. This is difficult to assess because although both filters increased FXIIa antigen, this assay is influenced by MB. However, levels of FXIIa antigen in the final MB-removed component were not higher than untreated plasma units. The filtration times for one filter (Maco Pharma Blueflex) were longer compared with the other (Pall MB1) (5-11 vs. 2-5 min, respectively), but the loss of plasma was equivalent for both filters. However, there did not appear to be any difference between the two filters in terms of activation of the contact system or thrombin generation as evidenced by the generation of FXIIa or prothrombin *F*1 + 2. Our results using the MB1 filter compare well with that previously published,[14] showing minimal loss of clotting factor activity. However, there was an apparent increase in levels of fibrinogen and PT-derived coagulation factors after filtration with the MB1 filter, which was associated with a small decrease in the PT ratio. We cannot explain these results because these assays do not appear to be influenced by the presence of MB, but they could possibly be a result of small increases in the activation state of coagulation factors. The changes in coagulation factors observed after filtration with either MB-removal filter appear to be clinically insignificant. Neither MB-removal filter resulted in generation of C3a des arg, a marker of complement activation. However, levels after filtration were reduced using the Pall MB1 filter. Whether this has any clinical benefit in terms of acute reactions is unclear.

As well as examining the loss of coagulation factors during MB photoinactivation, we compared residual levels in the final component with reference ranges based on previous studies of nontreated FFP in our laboratories. Despite the observed losses of coagulation factors due to MB treatment, final levels of all coagulation factors in frozen-thawed, MB-treated, and removed FFP were above the lower limit of the reference range in over 90 percent of units. However, over 50 percent of units were above the upper reference range for PT ratio. This presumably reflects the loss of fibrinogen and FII, FV, FVII, and FX because the PT is dependent upon these factors. However, the PT ratio of all units was less than 1.35. In addition, 23 percent of units had levels of prothrombin *F*1 + 2 higher than the upper limit of the reference range, reflecting the increase in prothrombin *F*1 + 2 seen due to the MB process. The clinical significance of increased *F*1 + 2 levels is unclear, but values higher than those observed in our study are seen in S/D-treated plasma.[23] We did not assess the effect of MB treatment or removal on plasma inhibitors of coagulation. However, MB treatment is reported to have minimal effect on levels of antithrombin, α_2-antiplasmin and protein C & S.[5,7,11] After MB removal (Pall MB1 filter), levels of antithrombin and protein C & S are within the normal range.[14]

We also evaluated the addition of a WBC-reduction step before freezing plasma, which did not appear to augment the loss of fibrinogen and FVIII activity. VWF:CP activity in plasma that was WBC reduced, MB inactivated, and removed using the MB1 filter was also within reference ranges established by other laboratories, suggesting that these processing steps do not have a major effect on VWF:CP using the techniques employed. These results are consistent with our previous findings on MB-treated fresh plasma[24] and others results on frozen-thawed plasma.[25] However, we cannot exclude small losses of activity given the relatively small number of samples used in this study.

In the UK, all blood components are WBC-reduced before storage. However, for logistical reasons we also wanted the flexibility to freeze non-WBC-reduced plasma intended for MB treatment, as a subsequent WBC-reduction step is integral to the Maco Pharma MB process. We were concerned that freeze-thawing may result in reduced cell removal by the WBC-reduction filter or cause fragmentation of cells with the potential for increasing the risk of transmission of vCJD. Most cellular prion protein in blood, used here as a surrogate marker for the potentially infective abnormal prion protein, is associated with

plasma (65%), with 26.5 percent, 1.8 percent, and 0.8 percent associated with platelets, RBCs, and polymorphonuclear cells, respectively.[13] Our results indicate that the majority of WBCs can be detected after thawing, and these are removed to undetectable levels after the WBC-reduction step of the MB process. However, the method we employed to detect WBCs predominantly measures WBC nuclei (unpublished data), and therefore provides little information on cellular integrity. The majority of WBCs in freeze-thawed plasma are detectable with PI without prior permeabilization,[26,27] suggesting that freeze-thawing alters WBC membrane integrity. The increase in levels of α_1-proteinase inhibitor: PMN elastase complexes after thawing of plasma spiked with WBCs shows that PMN degranulation is occurring, but the postthaw levels remain below 100 µg per L, which is not suggestive of large-scale PMN disintegration. Furthermore, in the absence of platelets, levels of LDH did not increase substantially after freezing, suggesting that WBCs do not disintegrate. We were unable to assess WBC fragments due to the insufficient sensitivity of available methods.

When platelets were spiked into plasma, there was an increase in platelet-derived microparticles after freeze-thawing of plasma, which probably explains the small decrease in platelet count detected by flow cytometry because these events would not be included in the platelet count. This fall was not detected by hematology analyzer, possibly because cell fragments can be detected as platelets by impedance-based methods. These fragments were reduced to or below the level in WBC-reduced fresh plasma after the WBC-reduction step of the MB process. However, we also analyzed cell microparticles based on the binding of purifed annexin V, which has a high affinity for anionic phospholipids. Freeze-thawing resulted in an increase in annexin-V-positive microparticles, which appear to be mainly derived from platelets and were only partially removed by WBC reduction. The increased detection of microparticles by this method compared with using an antibody against the platelet receptor CD61 is probably attributable to the greater number of molecules per platelet of anionic phospholipid (1×10^6) compared with CD61 ($4\text{-}8 \times 10^4$).[28,29] The presence of RBC and WBC microparticles (which will also bind annexin V) may also help to explain this difference, but this seems unlikely because in the absence of platelets the differences between methods were less pronounced. The number of annexin-V-positive microparticles found in non-WBC-reduced plasma that has been frozen-thawed and then filtered is not appreciably higher than would be found in plasma that we currently produce.

The effect of loss of coagulation factor activity due to MB treatment on the in vivo efficacy of the component is difficult to assess because there are no published randomized, controlled clinical trial data comparing MB to either standard FFP or S/D-treated FFP. However, 2.5 million units of MB FFP have been transfused internationally without obvious clinical sequelae.[30,31] In Spain, the switch from standard to MB-treated FFP has been associated with an increase in demand for FFP and cryoprecipitate,[32] which the authors attribute to loss of coagulation activity. However the increase in use (56%) appears to be disproportionate to the decrease in coagulation factors, suggesting that other factors, such as perception of a safer component, may have been influencing usage. It is also reported that the use of MB FFP is associated with a higher number of plasma exchanges compared with untreated FFP for the treatment of thrombotic thrombocytopenic purpura,[33] although we found no difference in the levels of VWF cleaving activity, the presumed therapeutic moiety in plasma treatment of thrombotic thrombocytopenic purpura, in MB FFP.[24] It is critical that transfusion services introducing pathogen inactivation of components monitor ongoing trends in usage as well as having a system for hazard reporting. At the time of writing, MB-treated and -removed FFP is routinely produced in England and Wales for transfusion to children and neonates born after 1995, with similar arrangements in other parts of the UK. However, in the near future, plasma to be pathogen inactivated for this patient group throughout the UK will be imported from volunteer donors in North America. Processes currently available for the pathogen inactivation of plasma all result in a decrease in coagulation factor activity. Improvements in the safety of blood need to be balanced against some likely reduction in the component potency. Single-unit systems for pathogen inactivation of plasma that have less effect on coagulation factor activity are clearly desirable.

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