

TRANSFUSION

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Evaluation of the haemostatic capacity of methylene blue treated liquid (not frozen) plasma stored up 14 days at 2° to 6°C.

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6 **Evaluation of the haemostatic capacity of methylene blue treated liquid (not frozen)**
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8 **plasma stored up 14 days at 2° to 6°C.**
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11 Short running headline: Liquid methylene blue treated plasma
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14 Authors :
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16
17 André Rapaille¹, Anaïs Lotens¹, Nicolas de Valensart¹, Tome Najdovski¹, Laura Green²
18

19
20 ¹ Service du Sang, Belgian Red Cross, Suarlée, Belgium
21

22
23 ² Queen Mary University of London, NHS Blood and Transplant and Barts health NHS Trust, London
24
25 UK
26

27
28 Corresponding author:
29

30
31 André Rapaille
32

33
34 e-mail address : andre.rapaille@croix-rouge.be
35

36
37 Telephone number: +3281564261
38

39
40 Fax number: +3281564214
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55 CONFLICT OF INTEREST
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58 The authors have no conflict of interest to declare.
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5 **BACKGROUND:** Early plasma transfusion for management of bleeding, particularly trauma,
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7 is associated with better outcomes. Improving the availability/safety of plasma transfusion for
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9 patients is essential for transfusion services. The aim of this study is to evaluate the
10
11 haemostatic capacity of methylene-blue (MB) liquid (not frozen) plasma over time.
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13 **MATERIALS AND METHODS:** Twenty whole blood-derived plasmas units collected from male
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15 donors were separated and processed within 18 hours of collection. Individual plasmas were
16
17 treated with MB and stored in liquid status at 2 – 6°C for 14 days. A range of coagulation
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19 assays, including thrombin generation, rotational thromboelastometry (ROTEM) and
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21 Thrombodynamics were tested at different time-points, together with bacterial growth.
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23 **RESULTS:** Apart from Factor (F)XII, other coagulation factors (fibrinogen, FV, FVIII, FXI)
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25 reduced significantly after MB treatment, with levels remaining stable except for FVIII
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27 afterwards. By day 14, most clotting factors were >0.7 IU/mL, apart from FVIII. There was a
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29 disproportionate decrease in Protein S (PS) activity compared to free PS antigen and by day
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31 14 its value was ~50%. There was no significant difference in maximum clot formation
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33 (ROTEM) and clot-density (Thrombodynamics) over time. Endogenous thrombin potential
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35 (Thrombin-Generation), clot-size and velocity-index (Thrombodynamics) decreased
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37 significantly over time consistent with clotting factor reduction. There was no bacterial growth.
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39 **CONCLUSIONS:** MB-treated liquid plasma stored at 2-6°C can be used for up to 14 days: the
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41 long shelf-life, the liquid status and the MB treatment will improve its availability for
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43 management of bleeding as well as providing a safe component from pathogens.
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49 Key Words: Liquid plasma, Methylene Blue, Pathogen Inactivation, Haemostasis
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INTRODUCTION

Several studies have now shown that management of major bleeding associated with trauma with early plasma transfusion, for both hospital and pre-hospital care, improves survival¹ and reduces death due to exsanguination². Most countries use fresh frozen plasma (FFP) components to resuscitate bleeding patients in emergencies, however, one of the main limitations of frozen products is the need for thawing, which can take between 20 – 30 minutes depending on the thawing devices used³⁻⁶. Being able to store thawed FFP for extended periods at 2° to 6°C is one way to improve the availability of plasma in emergency settings, and some countries have already implemented this practice by extending the shelf life of thawed plasma for up to 120 hours^{7,8}. However, in vitro studies have shown that the coagulation factor in extended thawed FFP decline over time, and impairment in the quality of plasma over time must be balanced against the clinical need for making plasma available quickly for treatment of bleeding⁷.

Liquid plasma (LP) that has never been frozen, is another option that has been used in some countries, with its shelf life being from 7 to 14 days⁹⁻¹¹ in Sweden, and between 26 to 40 days in the United States, depending on the manufacturing process¹². Like extended thawed FFP, in vitro data also show reduction in potency of LP components over time in terms of coagulation factors¹³.

Another important aspect of plasma preparation is the pathogen inactivation (PI) step, which in many European countries is a mandatory process. PI of plasma also induces a decrease in coagulation factors, which varies depending on the PI method used¹⁴. Most studies have reported on the quality of PI plasma that has been previously frozen and stored for up to 7 days after thawing, using either methylene-blue (MB) treatment¹⁵⁻¹⁸, solvent-detergent plasma (SDP)^{19,20}, or amotosalen/UVA light treatment²¹. Unlike above studies, in this work we evaluate the haemostatic capacity of liquid plasma (i.e. not frozen) treated with MB and stored for up to 14 days at 2° to 6°C, with regards to coagulation factors stability and global clotting activity.

MATERIALS AND METHODS

Plasma collection and processing

Whole blood units were collected in “Top and Bottom” configuration quadruple bags sets (Composelect®, Fresenius Kabi, Bad Homburg, Germany) with 63 ml of CPD anticoagulant. Whole blood-derived (WB) plasmas were separated after whole blood storage on eutectic plates at 20°C and immediately processed within 18 hours from collection. The centrifuged units were processed in accordance with standard operating procedures to obtain Red Blood Cells Concentrate, Buffy-Coat and Plasma (Maco Press, Macopharma, Tourcoing, France). Plasma selection involves gender (male donors) and volume. Male donors were selected to reduce the risk of transfusion-related acute lung injury. By visual in-process control, lipaemic or haemolytic specimens were excluded from the MB process. Twenty individual plasmas were treated using the Macopharma THERAFLEX® MB-Plasma Bag system with PLAS4 filter and Bluexflex filter (Macopharma, Tourcoing, France). Plasma units were connected to the THERAFLEX MB-Plasma kit and filtered by gravity. The 85µg anhydrous MB chloride in the form of a dry pill was sufficient to obtain an approximate concentration of 1 µM for a plasma volume of plasma between 230-318 mL. The pill in the transfer line is dissolved in the plasma during transfer. The packs were illuminated using the MacoTronic B2 (Macopharma, Tourcoing, France) plasma illumination system. The peak emission wavelength for the lamps was 630 nm. This procedure delivers the required dose of 120 J/cm². Illuminated plasmas were filtered on Blueflex filter to eliminate at least 90% of MB and its photoresidues. The whole procedure was performed in accordance with the manufacturer’s instructions. All plasmas were stored in cool chamber at 4°C for 14 days.

Plasma sampling

A sample poche (VSE0000Y Macopharma, Tourcoing, France) was attached to the storage bag by using a sterile connection device (TSCD model SC-201, TerumoBCT). For each

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3 plasma unit, samples were taken just before MB-treatment (T0), immediately after treatment
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5 (Day 1), and on days 3, 5, 7, 9, 11 and 14 post MB treatment for *in vitro* parameter analysis.
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7 Samples were divided in aliquots of 0.5 ml and were snap-frozen within 30 minutes and stored
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9 at a temperature below -70°C until they were tested.
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11 12 *Residual Red blood cells (RBC), white blood cells (WBC) and Platelets*

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15 Residual RBCs and platelets were measured using the plasma count kit (Becton Dickinson)
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17 and low-level WBCs using the leucocount kit (Becton Dickinson) on a flow cytometer
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19 (FACVia,® Becton Dickinson). The limits of detection for each assay were RBCs, $0.5 \times 10^6 / L$;
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21 WBCs, $0.1 \times 10^6 / L$; and PLTs, $73 \times 10^6 / L$.
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23 24 *Coagulation factors assays*

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27 Plasma samples were thawed in a 37°C water bath to proceed with coagulation tests. **The**
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29 **Clauss method was used with HemosIL reagents to determine fibrinogen concentration.** The
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31 following coagulation assays were performed on the ACL TOP 700 (Werfen, Barcelona, Spain)
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33 using HemosIL® reagents FV/FVIII/FXI/FXII Deficient Plasma): activated partial
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35 thromboplastin time (APTT), one-stage factor (F) V, FVIII, FXI, and FXII. Coagulation factor
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37 assays were standardized using HemosIL Calibration Plasma (Werfen) which is calibrated
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39 against the WHO standard. For the quantification of **free** protein S (PS) **antigen**, latex particles
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41 coated with purified C4BP and latex particles coated with monoclonal anti-PS were added to
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43 the unknown plasma sample along with albumin C4BP buffer (HemosIL Free Protein S,
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45 Werfen). Functional protein S was measured using the HemosIL Pro S reagent kit (HemosIL
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47 Protein S Activity, Werfen) on an ACL TOP 500 coagulation analyzer (Werfen) The first
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49 international standard for protein S plasma, human (WHO-93/590), served as the plasma
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51 standard while the control plasma was contained within the HemosIL Pro S kit.
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54 55 *Rotational thromboelastometry*

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3 Rotational thromboelastometry (ROTEM Delta; Werfen) was performed with a method adapted
4 from Sorensen et al²². In brief, 20 µL of starting reagent (0.2 mol/L calcium chloride in 20
5 mmol/L HEPES-buffered saline, pH 7.35) and 20 µL of tissue factor/synthetic phospholipids
6 reagent (1/1000 dilution in Owren's Veronal buffer of Dade Innovin Reagent, Siemens,
7 München, Germany) were mixed and followed by the addition of 300 µL plasma. The
8 subsequent reaction was monitored for 60 minutes. The following ROTEM parameters relating
9 to thrombus formation were studied: time to initial clot formation (CT) and maximum clot
10 firmness (MCF).
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20 21 *Thrombin generation*

22 The thrombin generation assay (TG) was performed using a calibrated automated
23 thrombogram system (Stago, Asnières-sur-Seine, France) in conjunction with the
24 manufacturer's PPP Reagent and PPP Reagent low, which have concentrations of
25 approximately 5 and 1 pmol/L tissue factor, respectively and 4 mmol/L of phospholipid mixture.
26 The exact concentrations are not disclosed by the manufacturer (Stago). A dedicated software
27 program (Thrombinoscope bv, Maastricht, The Netherlands) enabled the calculation of
28 thrombin activity against the calibrator (Thrombin Calibrator, Stago) and displayed the
29 following thrombin generation parameters: lag time, peak thrombin, endogenous thrombin
30 potential (ETP) corresponding to the area under the curve, and the velocity index (VI)
31 measured as peak / (time to reach peak-lag time).
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44 45 *Fibrin generation*

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47 Thrombodynamics (Hemacore, Moscow, Russia) measures and analyses the spatiotemporal
48 dynamics of fibrin clot growth in a heterogenous in vitro system. The coagulation process starts
49 from a localized surface which has immobilized tissue factor mimicking blood vessel wall
50 damage. The 4D PLS kit is used in order to perform and to correlate the spatiotemporal
51 dynamics of fibrin clot formation with the thrombin generated in the different plasma samples.
52 The biochemical reactions of the coagulation cascade and clot formation process are
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3 characterized by measurements of clot size (CS) and calculation of spatial clot growth rate
4 (initial rate, Velocity Index, Vi) along with the clot density (CD). The Thrombodynamics assays
5 would be sensitive to hypo- and hyper coagulate state, because spontaneously formed fibrin
6 clots can also be detected.
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11 *Sterility testing*

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15 Bacterial growth was tested in all plasma units after 14 days of storage by incubating 10 mL of
16 each bag under aerobic and anaerobic conditions at 36°C for 7 days with BacT Alert 3D
17 (bioMérieux).
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21 *Statistical analysis*

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25 The results of tests are expressed as mean \pm standard deviation. Statistical analysis was
26 performed using computer software (Minitab software, Minitab Inc.). Normality and statistical
27 significance were tested for each test variable. Statistical analyses were performed with paired
28 Student's t-test between T0 and day 1 to study the MB treatment effects on the plasma
29 parameters studied. Analysis of variance (one-way ANOVA) with Tukey's multiple
30 comparisons test was used to compare storage data of coagulation parameters between Day
31 1 to Day14. Cuzick test was used to determine if there is a statistically significant linear trend
32 of reducing global tests results over time. A p-value of <0.05 indicates significant statistical
33 difference.
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48 **RESULTS**

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50 A total of 20 units male liquid plasma was collected which were blood group O (n = 9), A (n =
51 8) and B (n =3). After MB treatment, levels of WBCs and PLTs were below the limits of
52 detection in all units ($<1 \times 10^6$ and 5×10^9 /L, respectively). Levels of RBC contamination were
53 on average 0.05×10^9 /L and were below the current maximum amount of 6×10^9 /L recommended
54 in the Council of Europe guidelines²³.
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3 At the end of the storage, all bacterial cultures were negative.
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6 *Effects on coagulation factors*

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9 Results of coagulation factor over 14 days are shown in Table 1. With the exception of FXII,
10 all other coagulation factors (fibrinogen, FV, FVIII, FXI) reduced after MB treatment. Over time,
11 the reduction in coagulation factors, fibrinogen, FV, FXI, FXII, levels was not significant. FVIII
12 decrease significantly between day 1 and day 3. After that FVIII levels stayed stable over the
13 time (Tukey comparison test). It is important to emphasise that by day 14, apart from FVIII
14 levels, all other factors were >0.65 IU/mL. The free PS antigen did not change significantly
15 over time, however PS activity decreased over time ($p<0.001$), and by days 7, 11 and 14 the
16 values were > 70%, >50% and just under 50%, respectively.
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25 *Global coagulation assays*

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28 The APPT time increase significantly after MB treatment ($p<0.001$). The APPT time was more
29 prolonged between Day 1 (35.9 +/- 2.3 seconds) and Day 3 (39.7 +/- 3.4 seconds) (Tukey
30 comparison test), but after Day 3 the APPT time was not lengthened. Result of ROTEM and
31 fibrin generation are shown in Table 2, while thrombin generation results are given in Figure
32 1. There was an increase in CT for ROTEM from 10.8 (+/-1.3) minutes in day 0 (T0) to 19.8
33 (+/-4.6) minutes in Day 1 after MB treatment. Over the time the CT increase up to 27.7 (+/-
34 5.2) minutes in day 14 (Cuzick test, $p<0.001$). However, there was no significant difference in
35 MCF over 14 days (Cuzick test, $p=0.732$).
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46 As far as thrombin generation parameters are concerned results showed that the lag time
47 increased significantly at 1 pmol/L tissue factor concentrations (Cuzick test, $p<0.001$), did this
48 was not the case with 5 pmol/L tissue factor concentrations (Cuzick test, $p=0.219$). Peak
49 thrombin and velocity index increased at Day 1 compared to Day 0 (t test, $p<0.001$), and after
50 that both parameters reduced over time with both tissue factor concentrations (Cuzick test,
51 $p<0.001$). ETP reduced with both tissue factor concentrations (Cuzick test, $p<0.001$).
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3 Fibrin generation test using Thrombodynamics device, showed a decrease for the clot growth
4 rates (Vi) (Cuzick test, $p=0.005$). Clot densities (CD) stayed stable over the time (Cuzick test,
5 $p=0.486$), while clot size (CS) reduced significantly between day 1 to day 14 (Cuzick test,
6 $p<0.001$).

15 DISCUSSION

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18 In this study we describe haemostatic changes over time (up to 14 days) in liquid plasma that
19 has been treated with methylene-blue (MB). The key findings to our study are 1st) as far as
20 white cells, red cell and platelets are concerned, all results were within the Council of Europe
21 specification; 2^d) except for FXII, all clotting factor reduced after MB treatment. Over time, the
22 decline was more significant for FVIII and PS activity; most factors remained stable. 3^d) by day
23 14, apart from FVIII and PS activity, all clotting factors were at ~70% activity and 4th) Thrombin
24 generation showed reduction in peak thrombin and endogenous thrombin potential (consistent
25 with above clotting factors changes). Rotem clot time, fibrin generation clot growth rate and
26 clot size showed a decrease over time; while ROTEM maximum clot firmness (MCF) and
27 Thrombodynamics clot density were overall stable.

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30 Several factors can impact on the haemostatic changes of liquid plasma in our study, but two
31 most important ones that will be discussed below are a) PI treatment with MB and b) storage
32 condition. The effect of MB treatment on coagulation factors in plasma has been previously
33 described by several studies, with most evaluating plasma that had been previously frozen and
34 thawed for up to 24 hours^{15,24-29}. All these studies showed that most of the clotting factors
35 reduced following MB treated plasma.

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38 Unlike above studies, in this work we evaluated MB-treated liquid plasma that was stored for
39 up to 14 days, which makes it difficult to compare our data with above studies, as it is well
40 known that freezing/thawing process can impact haemostasis of plasma⁷. However, common
41 themes between other studies^{15,24-29} and ours are that MB-treatment reduces most clotting

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3 factors. Beyond three days, and by day 14, most clotting factors remained stable and were at
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5 ~70%, apart from FVIII and PS activity. It is well known that the most affected clotting factors
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7 by the MB-treatment are FVIII, fibrinogen and FXI, where losses have been reported to be
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9 approximately 30–40%³⁰. In addition to the MB treatment, FVIII levels are also sensitive to
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11 storage conditions, and blood groups^{16,31-34}. In our study 45% of plasma components collected
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13 were from blood group O donors (known to have lower FVIII levels than non-group O), and
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15 despite this, the FVIII levels were quite comparable with above studies, with its recovery being
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17 89%. Unlike FVIII, in our study fibrinogen level reduction was not significant, indicating minimal
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19 losses through the whole process, which we believe to be due to not having to freeze and thaw
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21 the plasma. Further, FVIII and fibrinogen recovery at day one (86% and 84% respectively) after
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23 MB treatment in our study are consistent with published data^{29,33}.

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27 FXI is one of a contact factors that contributes to haemostasis by activating FIX. By day one
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29 after MB treatment, FXI recovery in our study was 80%, which is consistent with data published
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31 by Larrea²⁹ (81%). Early publications from Hornsey et. al.²⁴ has shown a higher FXI recovery
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33 (87%), and we believe that the slight difference seen between Hornsey and our study and
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35 Larrea's study, could be due to the timing when plasmas are separated after a whole blood
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37 donation and timing of PI treatment which are different between studies.

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41 One of the main concerns with storing liquid plasma at 4°C has been the increase in contact
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43 activation seen over time with storage, which is more pronounced in women than men^{7,11,13} :
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45 this has resulted in countries restricting the shelf life of liquid plasma to seven days^{11,13}. In our
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47 study however, we saw no increase in FXII or FXI, and both factors remained stable over time.
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49 We believe that by using male only donors for the MB-liquid plasma, we have reduced this
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51 effect, which will thus allow us to consider a longer than 7 days shelf life for this component.

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54 Another potential benefit of using male donor for plasma is the protein S levels, which are
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56 higher in men than women. Like many other studies^{7,13}, we saw a disproportionately higher
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58 loss for PS activity compared to free PS antigen. In the past, low levels of protein S activity
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60 and antiplasmin in solvent-detergent treated plasma have resulted in increased risk of

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3 thrombotic complications^{35,36}. However, the solvent detergent plasma produced then, had
4 almost completely absent PS activity, which is not the case at all with MB liquid plasma studied
5 here. Several studies have demonstrated no impact on the discordance between free protein
6 S antigen and protein S activity with MB treatment^{17,37}, while one reported a small difference³⁸.
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8 However, studies of liquid plasma stored at cold temperature have shown disproportionate
9 decrease in PS activity compared with the loss of free PS¹³. Based on these we can speculate
10 that the underlying biology could be due to cold-storage effect, although we don't understand
11 the mechanism behind it. Further, all global clotting tests used in this study to evaluate the
12 quality of plasma showed no increase in thrombin potential (for thrombin generation assay),
13 maximal clot formation (ROTEM) or clot density (fibrin generation test), confirming that plasma
14 is not thrombogenic.

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16 The optimal levels for all clotting factors required to make the treated plasma efficacious for
17 treatment of bleeding (the most common indications for plasma transfusion), remains
18 unknown^{7,30}, which makes it very hard for any blood services to determine the most appropriate
19 shelf-life for the plasma components. However, learning from past experiences and putting
20 together our results in the context of other study results, we can conclude that as far as the
21 shelf-life of MB-treated liquid plasma evaluated in this study is concerned, the shelf-life of the
22 components can be as long as 14 days, which for bleeding patients could be of immense
23 benefits as it will improve the availability of plasma for management of bleeding, as well as
24 improve the logistics of managing blood stocks in hospitals or blood services, and provide a
25 component that is safe from different pathogens.

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7 Aquitaine, France) for performing Prot S Activity assays and Alain Guillet (SMCS-LIDAM, UCLouvain)
8 for performing Cuzick test.
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TABLE 1. Effect of cold (2-6°C) storage of MBLP on factors of coagulation system.

Variable	T0	Day 1	Recovery % †	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14	p value §
APTT (sec)	33.6 (± 2.5)	35.9(±2.3) *	107(±3)	39.7(±3.4)	40.0(±2.9)	40.1(±4.0)	39.6(±2.7)	39.7(±2.9)	39.8(±3.1)	p<0.001
Fibrinogen (g/L)	2.35(±0.50)	1.93(±0.34) *	83(±10)	1.88(±0.35)	1.80(±0.33)	1.96(±0.40)	1.87(±0.30)	1.89(±0.33)	1.89(±0.36)	p=0.872
FV (IU/mL)	0.86(±0.18)	0.81(±0.18) *	95(±6)	0.74(±0.17)	0.75(±0.17)	0.73(±0.16)	0.67(±0.15)	0.68(±0.15)	0.69(±0.14)	p=0.073
FVIII (IU/mL)	0.98(±0.21)	0.84(±0.19) *	86(±7)	0.59(±0.15)	0.55(±0.15)	0.50(±0.15)	0.49(±0.14)	0.51(±0.13)	0.52(±0.15)	p<0.001
FXI (IU/mL)	0.95(±0.14)	0.76(±0.14) *	80(±10)	0.71(±0.12)	0.69(±0.12)	0.67(±0.12)	0.65(±0.11)	0.67(±0.12)	0.69(±0.13)	p=0.096
FXII (IU/mL)	1.17(±0.45)	0.97(±0.16)	88(±14)	0.94(±0.16)	0.88(±0.17)	0.81(±0.13)	0.91(±0.28)	0.85(±0.14)	0.87(±0.14)	p=0.094
Free PS (%)	94.9(± 17.7)	91.9(±18.7) *	97(±4)	92.5(±17.8)	90.0(±17.0)	88.9(±15.7)	89.8(±16.2)	86.9(±15.7)	87.7(±15.9)	p=0.936†
PS activity (%)	94.9(±20.6)	87.9(±19.5) *	93(±3)	79.2(±23.2)	72.0(±21.8)	74.0(±21.7)	62.9± (20.1)	55.1(±19.1)	46.6(±18.8)	p<0.001

Data are reported as mean (±SD); n=20

* Significant difference (paired t' test) between T0 and D1 values

† Recovery between D1 and T0

§ one-way ANOVA between D1up to D14 storage

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TABLE 2. ROTEM and Thrombodynamics analysis of LMBP

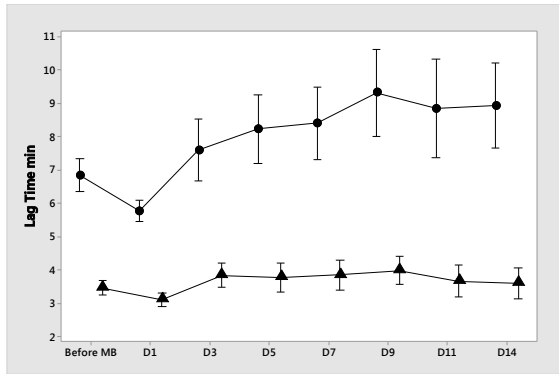
Variable	T0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14	p value ‡
CT (min)	10.8 (± 1.3)	19.8(±4.6) *	22.3(±4.3)	26.0(±7.1)	25.8(±5.4)	25.5(±6.5)	34.0(±14.8)	27.7(±5.2)	p<0.001
MCF (mm)	24.3(±6.2)	28.3(±6.3)	28.5(±7.4)	29.2(±7.1)	28.6(±6.9)	28.1(±4.8)	27.8(±6.0)	28.7(±5.1)	p=0.732
Vi (um/min)	64.0(±3.5)	54.7(±4.8) *	53.1(±4.1)	53.3(±4.0)	49.3(±11.0)	51.9(±2.7)	51.1(±3.6)	51.6(±4.1)	p=0.005
CD (a.u.)	11506(±2212)	10506(±2001) *	10271(±2006)	10247(±1571)	10792(±2230)	10591(±1851)	10792(±1841)	10584(±1771)	p=0.486
CS (µm)	1418(±81)	1294(±159) *	1179(±92)	1137(±240)	1101(±208)	1140(±99)	1118(±93)	1143(±124)	p<0.001

Data are reported as mean (±SD); n=20

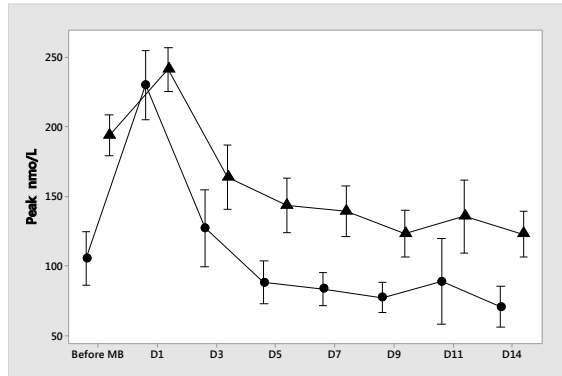
* Significant difference (paired t' test) between T0 and D1 values

‡ Cuzick trend test between D1 up to D14 storage

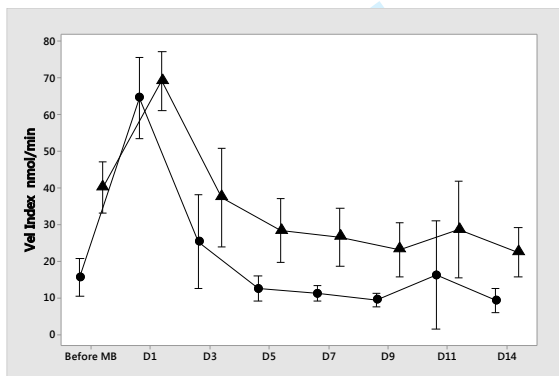
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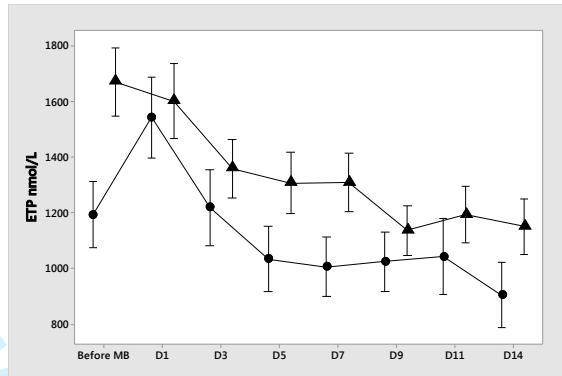
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5 Fig. 1. Thrombin generation results up to 14 days storage. 1pmol/L tissue factor, black circle; 5 pmol
6 /L tissue factor, black triangle. The data represent mean and 95% confident interval (error bars) from
7 20 LMBP. (A) Lag Time. (B) peak thrombin. (C) velocity index. (D) ETP.
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