Cold-stored leukoreduced CPDA-1 whole blood: in vitro quality and hemostatic properties

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BACKGROUND: Some jurisdictions require leukoreduction of cellular blood components. The only whole blood collection set with a platelet-saving filter uses citrate-phosphate-dextrose (CPD) as storage solution. Substituting CPD with citrate-phosphate-dextrose-adenine (CPDA-1) increases shelf life from 21 to 35 days. This would simplify prehospital and rural resupply and reduce wastage. We investigated in vitro quality and hemostatic properties of CPDA-1 whole blood leukoreduced with a platelet-saving filter.

STUDY DESIGN AND METHODS: CPDA-1 whole blood was leukoreduced using a platelet-saving filter and stored 35 days. EDQM requirements, hematology, metabolic parameters, thromboelastography, light transmission aggregometry, fibrinogen, factor VIII, and interleukin-6 were measured on Days 0, 1, 14, 21, and 35 and compared to non-leukoreduced blood.

RESULTS: All units met EDQM requirements. Leukoreduction yielded residual white blood cell count <1 x 10^6 and 87% platelet recovery on Day 1. It caused reduction in thromboelastography parameters, but not aggregometry response. No hemolysis >0.8% was observed. Factor VIII was higher on Day 35 in the leukoreduced group, 37.9 (95% CI: 26.0, 49.8) versus 13.8 (9.4, 18.2) IU/dL. In both groups, aggregation was significantly reduced by Day 14. Thromboelastography showed remaining platelet activity on Day 35, MA 46.9 (42.1, 51.7) in the leukoreduced and 44.3 (39.6, 49.0) mm in the non-leukoreduced group. Fibrinogen was within reference ranges at Day 35 (>2 g/dL). Interleukin-6 was not detectable.

CONCLUSION: Leukoreducing CPDA-1 whole blood with a platelet-saving filter did not compromise hemostatic properties. We encourage development of a single bag CPDA-1 whole blood collection set with in-line platelet-saving filter.

T he introduction of blood component therapy in the 1970s allowed blood banks to optimize the utilization of the collected whole blood. With component therapy, a single whole blood collection could benefit multiple patients. However, severely hemorrhaging patients require all components of blood—preferably in a physiological ratio with a minimum of hemodiluting additives.

Transfusion of multiple blood components simultaneously presents additional logistical challenges in a potentially chaotic setting. In prehospital and military far forward settings, these difficulties are enhanced by the fact that the individual components of the blood have different storage requirements. Because of this, and supported by the shift in hemostatic damage control resuscitation policies from clear
fluids to blood transfusion, recent years have seen a growing movement to reintroduce whole blood as the preferred resuscitation fluid for major hemorrhage.1,2

Regulations in many countries, including Norway, require that cellular blood components are leukoreduced3 to reduce the risk of transfusion-related immune modulation and transmission of viruses like cytomegalovirus. Leukoreduced whole blood was introduced in our local helicopter emergency medical service in December 2015 and at Haukeland University Hospital in December 2017. The whole blood is processed using a whole blood collection set with an inline platelet-saving leukoreduction filter and citrate-phosphate-dextrose (CPD) as the storage medium. Whole blood stored in this solution has a maximum shelf life of 21 days.4 There are multiple publications describing the in vitro function of whole blood leukoreduced with this system.5–12

Because of the complexity of resupplying far forward deployments with blood products, increased shelf life is a priority. Longer shelf life also increases availability of a platelet-containing blood component in civilian prehospital and rural services and enables early balanced blood transfusion to patients with life-threatening hemorrhage in locations where these occur less frequently. Commercial availability of a collection set featuring CPDA-1 as the storage solution and an inline platelet-saving leukoreduction filter could help address waste and supply concerns that prevent greater adoption of whole blood in prehospital and rural hospital settings. Research performed in the 1960s showed better preservation of adenosine triphosphate (ATP) synthesis in red cells if adenine was added to the anticoagulant.13–15 This enabled prolonged shelf life from 21 to 35 days for whole blood collected in citrate-phosphate-dextrose-adrenaline (CPDA-1).4

We have been unable to find publications that investigate in vitro quality and hemostatic properties of CPDA-1 whole blood leukoreduced with a platelet-saving filter. In this study, we therefore repurposed a platelet-saving leukoreduction filter used for CPD whole blood in order to study the impact of leukoreduction on CPDA-1 whole blood when stored cold for 35 days.

**MATERIALS AND METHODS**

**Study design and outcome measures**

The study was performed at Haukeland University Hospital in Bergen, Norway, with approval from the regional ethical committee (id: 2017/157). Healthy volunteer donors gave blood to the study after giving written informed consent.

The quality requirements for leukoreduced whole blood specified by the European Directorate for the Quality of Medicines & Healthcare (EDQM) (hemoglobin >43 g, residual white blood cell count <1 × 10^6, and hemolysis at expiration <0.8% of red cell mass16) were chosen as outcome measures. In addition, we measured blood cell counts, metabolic parameters, potassium, and interleukin-6 (IL-6). For evaluation of hemostatic capacity, we used coagulation markers, viscoelastic hemostatic properties, and platelet function.

**Blood collection**

Whole blood was collected using a collection set containing 63 mL of CPDA-1 (PB-1CD456M5S, Terumo BCT). This set can be used to collect 450 mL ± 10% of whole blood. We collected 490 mL of whole blood from each participant in order to have a post-sampling storage volume that approximates the 450 mL most commonly collected. The leukoreduction group (LR) and the non-leukoreduced control group (NONLR) each consisted of eight donors. The blood was processed after a 2-hour resting period. In the LR group, a storage bag with inline leukoreduction filter (Imuflex WB-SP BB*LGQ456E6 primary bag, Terumo BCT) was sterile welded to the CPDA-1 collection bag. The product was then leukoreduced according to the manufacturer’s manual. In the NONLR group, the same type of storage bag without filter was sterile welded to the CPDA-1 collection bag and the blood transferred by gravity. This was done to ensure that blood in both groups were stored in the same type of bag. After processing, the blood bags were stored at 4 ± 2°C without agitation.

**Blood sampling**

The first 4 units in each group were sampled on Days 0, 1, 8, 14, 21, 28, and 35. We considered 14, 21, or 35 days as the most relevant potential shelf lives. Because of this, we chose only to perform sampling on Days 0, 1, 14, 21, and 35 for the last 8 units. Before making this change, we collected and sampled 6 units only on Days 0, 1, and 35 to evaluate whether a reduction in sample points would affect the test results.

Sampling on Day 0 was performed prior to leukoreduction in the LR group. At each sample point, the primary bag was mixed gently with a head-over-heel motion and 27–52 mL (depending on volume required for analysis) transferred to a 150 mL transfer bag (Teruflex BB*T015CM, Terumo BCT). Because approximately 50 mL is retained in the filter used, the same volume was removed from the NONLR group on Day 0 to ensure both groups had similar volumes during storage.

**Analyses**

Hemoglobin (HGB), hematocrit (HCT), red blood cell (RBC) count, mean corpuscular volume, mean platelet volume, and white blood cell count (WBC) were analyzed in K2EDTA on a hematology analyzer (Cell-Dyn Sapphire, Abbott Diagnostics). Hemoglobin in plasma was measured using a photometer (HemoCue Plasma/Low Hb, HemoCue AB). Residual WBC count (rWBC) after leukoreduction was analyzed by flowcytometry (BD LeucoCount/BD FACSCanto II, BD Biosciences). pH was measured on a blood gas analyzer (ABL825 FLEX, Radiometer Medical ApS). Glucose, lactate, sodium, and potassium levels were measured on a chemistry analyzer (Cobas 8000 iSE/c702 module, Roche Diagnostics GmbH). The coagulation factors fibrinogen and factor
VIII were analyzed on a coagulation analyzer (STA-R Evolution/STA-R Max using STA-Liquid Fib/STA-Fibrinogen 5, STA-Deficient VIII, STA-PTT Automate 5, STA-CaCl₂ [0.025 M] and STA-Unicalibrator, Stago S.A.S).

The viscoelastic hemostatic properties time to first clot formation (R), rate of clot formation (α), and maximum clot strength (MA), was measured with a thromboelastograph (TEG 5000, Haemonetics Corporation). Briefly, 1 mL of blood was activated with 40 μL of kaolin and 340 μL transferred to a plain cup containing 20 μL of 0.2 M CaCl₂. The analysis ran until 30 minutes after reaching MA.

Platelet function defined as maximum aggregation (MaxA) and rate of aggregation (slope) was measured using light transmission aggregometry (LTA) (Chrono-log Model 700, Chrono-log Corporation) according to consensus guidelines published by the International Society on Thrombosis and Haemostasis (ISTH). Platelet-rich plasma (PRP) was prepared by centrifuging 15-40 mL of blood at 200 g for 10 minutes. After removal of PRP, the remaining volume was centrifuged at 1500 g for 15 minutes to yield platelet-poor plasma (PPP). The platelet count in PRP was measured to verify that it was within 150-600 × 10⁹/L. PPP and PRP was visually inspected for hemolysis and lipemia. Volumes of 250 μL of PRP and 500 μL of PPP were incubated at 37°C for 3 minutes and then transferred to the measuring wells. Under constant stirring at 1000 RPM and 37°C, PRP was activated with 10 μM ADP or 30 μM TRAP-6 (Roche Diagnostics GmbH) and left to run until maximum aggregation was reached. Each sample was run in two parallels, and the result was reported as the mean of these. Additionally, spontaneous aggregation was measured.

IL-6 concentrations were quantified by immunoassay covering the range 3.13-300 pg/mL (Human IL-6 Quantikine ELISA Kit cat. no. D6050/Immunossay Control Group 1 cat no. QC01-1, R&D Systems, Inc.). Plasma for analysis was prepared by centrifuging whole blood for 15 minutes at 2500 g. The supernatant was then centrifuged for 10 minutes at 10,000 g and frozen at −70°C. ELISA analysis was performed according to the manufacturer’s instructions. All samples, controls, blanks, and standards were analyzed in duplicate. Absorbance was measured at 450 nm with wavelength correction at 540 nm (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments, Inc.).

**Statistical analysis**

Results were reported as mean with 95% confidence intervals. The effect of storage time and leukoreduction on each outcome measure was investigated using a linear mixed effects model with time, study group, and their interaction as predictors. In the time domain, we used simple contrasts where the outcome measures at each sample point were compared to the baseline sample taken on Day 0. The interaction between time and study group was used to describe potential effects of leukoreduction on the outcome measures. The same model was used for comparing pre- and post-leukoreduction (Days 0 to 1) and 35-day storage (Days 0 to 14, 0 to 21, and 0 to 35), and the additional units sampled on only Days 0, 1, and 35. Statistical significance was considered for p values below 0.05. All analyses were performed using a statistical software package (R version 3.6.0 with NLME version 3.1-140, The R Foundation for Statistical Computing).

![Fig. 1. (A) Platelet count and (B) mean platelet volume in leukoreduced and non-leukoreduced CPDA-1 whole blood stored for 35 days. The leukoreduced group was filtered after sampling on Day 0. The interaction between storage time and study group in a linear mixed effect model is indicated with p values, which signify if there was a statistically significant difference between the two groups in how the measurements changed from Days 0 to 1, 0 to 14, 0 to 21, and 0 to 35. [Color figure can be viewed at wileyonlinelibrary.com]](image-url)
RESULTS

Immediate effects of leukoreduction

All units in the LR group were successfully leukoreduced to $<1 \times 10^6$ residual WBCs, fulfilling EDQM requirements. Some platelets were lost in the filtration process, representing an 87% (77, 98) recovery on Day 1 in the LR group compared to 98% (91, 105) in the NONLR group ($p = 0.041$). Leukoreduction also resulted in prolonged TEG R time, reduced TEG $\alpha$, and reduced TEG MA on Day 1, but the values remained within reference ranges for healthy subjects (Table S1, available as supporting information in the online version of this paper). No other variables, including LTA platelet function, were affected by leukoreduction (Table S1, available as supporting information in the online version of this paper).

Storage

Hematological parameters

As shown in Fig. 1A, both groups had a similar reduction in platelet count until Day 21, followed by an increase in the NONLR group. Hemolysis remained below 0.8% on Day 35, with no statistically significant difference between the groups. There were no changes in HGB levels or RBC count in either group. Hematocrit increased from 39.5 (47.6, 41.3) on Day 1 to 40.6 (38.5, 42.8) on Day 35 in the NONLR group and 36.9 (35.6, 38.4) to 38.1 (36.2, 40.1) in the LR group ($p < 0.001$).

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**Fig. 2.** (A) Glucose, (B) lactate, (C) potassium, and (D) pH in leukoreduced and non-leukoreduced CPDA-1 whole blood stored for 35 days. The leukoreduced group was filtered after sampling on Day 0. The interaction between storage time and study group in a linear mixed effect model is indicated with $p$ values, which signify if there was a statistically significant difference between the two groups in how the measurements changed from Days 0 to 1, 0 to 14, 0 to 21, and 0 to 35. [Color figure can be viewed at wileyonlinelibrary.com]
Similarly, MCV increased from 92.0 (89.7, 94.3) to 95.4 (92.9, 97.9) in the NONLR group and from 92.6 (90.0, 95.3) to 96.0 (93.5, 98.5) in the LR group (p < 0.001). MPV was stable the first 14 days, then decreased (Fig. 1B). White blood cell count in the NONLR decreased during storage (Table S1, available as supporting information in the online version of this paper).

**Metabolic parameters**

Detectable levels of glucose remained on Day 35 in both groups, with a slightly greater decrease during storage in the NONLR group (Fig. 2A). Lactate concentrations mirrored glucose consumption in both groups (Fig. 2B). The increase in potassium was greater in the NONLR group from Day 14 onward (p < 0.001) (Fig. 2C). pH levels remained above 6.4 throughout storage in all units (Fig. 2D). Complete data is available online as Table S1, available as supporting information in the online version of this paper.

**Thromboelastography and platelet aggregation**

From Day 1 onward, TEG MA in both groups decreased similarly and fell below the reference ranges for fresh blood samples on Day 35 (Fig. 3B). Leukoreduction caused a spike in TEG R on Day 1, with a return to similar values to the NONLR group by Day 14. TEG R remained within reference ranges at all sample points. The TEG alpha angle dropped below reference ranges on Days 21 and 35 in the NONLR group. The interaction between storage time and study group in a linear mixed effect model is indicated with p values, which signify if there was a statistically significant difference between the two groups in how the measurements changed from Days 0 to 1, 0 to 14, 0 to 21, and 0 to 35. Light transmission aggregometry could not be performed on Day 35 due to hemolytic platelet-rich plasma. [Color figure can be viewed at wileyonlinelibrary.com]
and LR group, respectively. When measured by LTA, the maximum aggregation responses to ADP and TRAP-6 were significantly reduced from Day 14. This trend was independent of leukoreduction (Fig. 3C, D). LTA could not be performed on Day 35 due to high levels of hemolysis in PRP. Increased spontaneous aggregation was observed during storage (Table S1, available as supporting information in the online version of this paper).

Fibrinogen and factor VIII
There was no statistically significant difference between the groups in how fibrinogen levels changed during storage (Fig. 4A). As shown in Fig. 4B, leukoreduction resulted in better preservation of factor VIII from Day 14 onwards, with 38 IU/dL (26.0, 49.8) in the LR group and 14 IU/dL (9.3, 18.2) in the NONLR group on Day 35 (p < 0.001) (Table S1, available as supporting information in the online version of this paper).

Interleukin-6
IL-6 concentrations were below 3.13 pg/mL in all units at all sample points.

DISCUSSION
In this study we have investigated the in vitro quality and hemostatic properties of CPDA-1 whole blood leukoreduced with a platelet-saving filter and stored cold for 35 days. Our findings show that the filter performs satisfactorily, with residual WBC counts well below EDQM requirements and similar to that previously published for CPD. We found no significant hemolysis on Day 35, indicating that the CPDA-1 storage solution does not cause changes to RBCs that result in them incurring more damage when passing through the filter. Furthermore, no differences were seen in HGB, RBC count, or HCT after filtration and throughout storage.

Leukoreduction resulted in some loss of platelets, but the loss was similar to that observed in routine leukoreduction of CPD whole blood with the same platelet-saving filter in our facility, and in previously published literature. The platelets in the leukoreduced blood were still viable after filtration, with no statistically significant difference from the non-leukoreduced blood in the reduction in aggregometry response from Days 0 to 1. TEG was affected by leukoreduction, but remained within reference ranges on Day 1. This is similar to what has previously been documented to be the case when filtering CPD whole blood.

With the exception of platelet count, only minor changes in hematological parameters were observed during storage. Hematocrit increased during storage in both groups. When seen together with the increasing MCV, it can likely be explained by swelling of RBCs. These changes were not statistically different between the groups. As seen in Fig. 1A, the platelet count in the NONLR group appears to increase from Days 21 to 35. This is an artifact caused by fragments from degraded cells in samples on Day 35 being incorrectly counted as platelets by the hematology analyzer.

Braathen et al. found that platelet concentrates that were deprived of glucose also demonstrated reduced TEG MA and aggregometry response. For whole blood, all units demonstrated remaining glucose on Day 35. The minor
differences in glucose levels between the groups is unlikely to be of any importance. The NONLR group demonstrated a greater increase in potassium than the LR group. This could be explained by the presence of more cells in the non-leukoreduced blood.

While there are no reference ranges for TEG in stored whole blood, the measurements in both groups on Day 21 remained within the reference ranges for fresh blood samples. Both platelets and fibrin contribute to the clot strength measured by the MA parameter, meaning that you will get some response even in the absence of platelets. The rule of thumb is that fibrin is responsible for around 20%-30% of the total MA. Siletz et al. found that if they removed the platelets from whole blood, MA fell to <20 mm. The >40 mm measurements we observed on Day 35 were high enough to suggest remaining platelet activity. These findings indicate that a certain amount of hemostatic potential remains in CPDA-1 whole blood on Day 35, whether filtration is performed or not. Whether this in vitro hemostatic potential translates to adequate hemostatic function in vivo has to be established through clinical trials. Such trials should also investigate whether the age of the blood has an effect on outcome.

Light transmission aggregometry is an optical method and therefore sensitive to interference from factors such as hemolysis in the platelet-rich plasma used in the analysis. Despite the low levels of hemolysis in the stored whole blood, the sample preparation of PRP and PPP for the analyses on Day 35 resulted in visible hemolysis that prevented analysis. The results do however show some remaining platelet function on Day 21. These observations support the TEG findings.

Fibrinogen is a critical factor in hemostatic resuscitation of patients with massive hemorrhage. As demonstrated both here and in previous studies, leukoreduction does not reduce fibrinogen levels. An interesting observation was that factor VIII levels were significantly higher in the leukoreduced group from Day 14 of storage. We did not perform further analyses to establish the potential mechanisms behind this, but this could be an interesting finding to investigate in future studies.

Interleukin-6 (IL-6) concentrations in non-leukoreduced platelet concentrates have previously been shown to increase during storage, and to correlate with occurrence of febrile non-hemolytic transfusion reactions. Heddle et al. found higher risk of febrile non-hemolytic transfusion reactions with an IL-6 level of 214 pg/mL. Previous studies on whole blood have found that IL-6 levels remain within normal physiological levels regardless of storage or leukoreduction. This is supported by our findings that the IL-6 concentrations never exceeded the detection limit of 3.13 pg/mL.

We conclude that leukoreducing CPDA-1 whole blood with a platelet-saving filter results in only minor differences to in vitro quality, and that leukoreduction can be introduced without compromising hemostatic properties. We encourage the development of a single-bag whole blood collection set with an in-line platelet-saving filter and CPDA-1 as the storage solution to improve availability of leukoreduced whole blood both in military and civilian pre-hospital services as well as in rural hospitals. We also encourage clinical studies that investigate if the age of the whole blood being transfused affects patient outcomes.

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CONFLICT OF INTEREST

The authors declare no relevant conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Summary of all measured variables in non-leukoreduced and leukoreduced CPDA-1 whole blood.