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In vitro Evaluation of DINCH-Plasticized Blood Bags for Red Blood Cell Storage with CPDA-1 Anticoagulant

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Keywords
DEHP · DINCH · Plasticizer · Phthalate · Red blood cells

Abstract

Introduction: Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly used in blood bags. Despite its protective effects on red blood cell (RBC) storage, concerns about its reproductive toxicity exist. This study investigated the in vitro quality of RBC concentrates stored in bags using di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) as an alternative plasticizer. ***Methods:*** Using a pool-and-split study design, we produced 20 matched homogenous quintets of RBC concentrates in two DINCH bags and three DEHP bags with citrate phosphate dextrose adenine (CPDA-1) anticoagulant. RBC storage quality was assessed weekly for 35 days. ***Results:*** On day 35, the median hemolysis levels in the DINCH bags (0.297–0.342%) were marginally higher ($p < 0.05$) than the DEHP bags (0.204–0.240%). All DINCH bags showed <0.8% hemolysis. RBCs in the DINCH bags showed increased mean corpuscular volume and decreased eosin 5′ maleimide binding than in the DEHP bags. Higher pO_2 and lower pCO_2 levels in the DINCH bags indicated better gas permeability than in DEHP bags. Other metabolic parameters were comparable in both bags. Compared to DEHP, DINCH exhibited considerably lower levels of plasticizer leaching into blood bags. ***Conclusion:*** The quality of RBC concentrates stored for 35 days in DINCH-plasticized blood bags with CDPA-1 is generally comparable to those in DEHP bags. Hence, DINCH can be a viable alternative to DEHP in blood bags for nonleukoreduced RBC storage even without the use of next-generation additive solutions to improve RBC preservation quality.

Plain Language Summary

A plasticizer is a chemical substance added to plastic to increase its flexibility. DEHP is a plasticizer that has been widely used in many products including plastic tubing and bags of medical devices. However, concerns about DEHP-related toxicity have been debated for many years. DEHP has been replaced with other plasticizers in many products, but it is still being used in blood bags due to its protective effect on RBC preservation. DINCH is an alternative plasticizer with a low toxicology profile. This study investigated the quality of RBC concentrates stored in blood bags using DINCH. Twenty sets of five RBC concentrates were produced using two DINCH bags and three DEHP bags with CPDA-1 anticoagulant, and the storage quality was assessed weekly for 35 days. On day 35, the median hemolysis levels in the DINCH bags (0.297–0.342%) were slightly increased than the DEHP bags (0.204–0.240%). However, all DINCH bags showed hemolysis lower than the regulatory limit of 0.8%. DINCH bags exhibited better gas permeability than DEHP

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bags. Compared to DEHP, DINCH exhibited considerably lower levels of plasticizer leaching into blood bags. Most of the other metabolic parameters were comparable in both bags. The quality of nonleukocyte-reduced RBC concentrates stored for 35 days in DINCH-plasticized blood bags with CDPA-1 is generally comparable to those in DEHP bags. Hence, DINCH can be a viable alternative to DEHP in blood bags for RBC storage, even without the use of next-generation additive solutions to improve RBC preservation quality.

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used to convert polyvinyl chloride (PVC) into a flexible and elastic material. DEHP has long been used to make various items commonly used in our everyday lives, such as plastic toys, cosmetic containers, and product-packaging materials [1]. It is also used to make medical products, including intravenous bags, tubing, and especially blood bags. The revolutionary introduction of DEHP for making blood bags in the 1950s significantly improved blood transfusion safety by preventing the breakage of blood bags and facilitating sterile manufacture and separation of blood components, leading to the complete replacement of conventional glass bottles [2]. DEHP is also unique in that it enhances red blood cell (RBC) stability during storage and prolongs its shelf life by increasing in vivo recovery and decreasing hemolysis [3]. It is unclear how DEHP reduces RBC deterioration, but it is believed that DEHP gets directly incorporated into the RBC membrane and stabilizes it [4, 5].

However, concerns about DEHP-related reproductive toxicity and potential endocrine dysfunction have been debated for many years [6, 7]. While DEHP-related reproductive toxicity has been identified in animal models, its safety in humans remains unknown [7, 8]. Following a 2002 safety assessment of DEHP for medical devices, the United States Food and Drug Administration (US FDA) recommended that non-DEHP alternatives can be considered for certain procedures (e.g., exchange transfusion, hemodialysis, total parenteral nutrition, and extracorporeal membrane oxygenation) in high-risk patient groups including male neonates, pregnant women carrying male fetuses, and peripubertal males [9, 10]. However, they also emphasized that not performing a necessary procedure because of the DEHP-associated risks may pose a greater danger to these patients. Some countries are legally restricting the use of DEHP for human products. France has banned DEHP tubings in hospitals for neonates, children, and maternity wards [9]. Europe will cease the exemption for using DEHP in blood bags and prohibit its use in all medical devices by May 2025 [11, 12]. South Korea has also banned its use in intravenous sets and restricts the amount of DEHP in blood bags to 150 ppm [13]. Because DEHP leaches from the bag to its contents [8], patients receiving blood transfusions are at risk of being exposed to DEHP. Many blood bags for platelet and plasma storage have successfully shifted from using DEHP to other plasticizers such as trioctyl trimellitate or butyryl trihexyl citrate (BTHC) [2]. However, replacing DEHP in RBC storage bags has been difficult because of its aforementioned beneficial and protective effects on RBC stability.

Transitioning to non-DEHP blood collection and storage systems without deterioration in blood component quality is an important goal for blood suppliers. Several previous studies have investigated potential substitutes for DEHP in RBC storage bags [9, 14–20]. Plasticizers such as di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH), BTHC, di(2-ethylhexyl) terephthalate (DEHT), di(2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTH), and 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester (DL9TH) have been evaluated under different conditions including different preparation methods, product volumes, storage, and additive solutions, with varying results. Unfortunately, blood product-manufacturing processes can differ greatly among countries. Many low- and middle-income countries have limited access to blood component-processing resources, including leukoreduction and apheresis [21]. A pilot study on nonleukoreduced RBC units reported that DOTH/DINCH and DOTH/DL9TH could be promising candidates to replace DEHP [20]. However, most of the studies on DEHP alternatives have been conducted in high-income countries with adequate resources, and there are not enough data in the literature on the validation of non-DEHP systems in diverse settings.

This study aimed to investigate the in vitro quality of nonleukoreduced RBC concentrates stored in DINCH-plasticized blood bags in citrate phosphate dextrose adenine (CPDA-1) anticoagulant solution without additive solutions. Using a pool-and-split study design, we manufactured and compared the quality of adult-sized RBC concentrates in two DINCH bags and three DEHP bags for up to 35 days of storage.

Materials and Methods

Blood Collection and Component Preparation

A pool-and-split study design was implemented to compare DINCH and DEHP blood bags (Fig. 1). Donors were recruited in accordance with the legal regulations for blood donation in Korea, with several modifications and additions made to the criteria, including age, the number of whole blood donations during the previous year, estimated blood volume, and unexpected antibody screening. Approximately 407 mL ± 10% of whole blood was

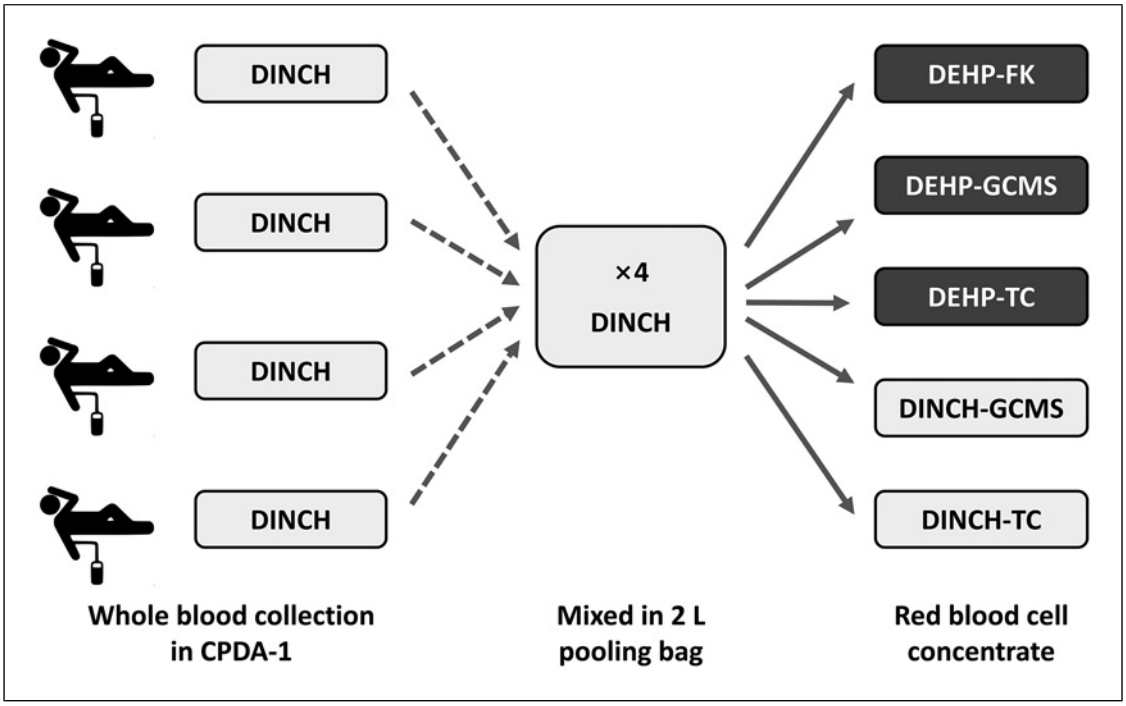

Fig. 1. Study design. A pool-and-split study was used to evaluate DINCH-plasticized blood bags for RBC storage with CDPA-1 anticoagulant. Whole blood collected from four donors was pooled and equally distributed into five blood bags to produce RBC concentrates. Twenty matched quintets of RBC concentrates were compared during 35 days of storage.

collected into non-DEHP prototype DINCH-plasticized blood bags (Green Cross Medical Science [GCMS], Yongin-si, South Korea) containing 57 mL of CPDA-1 anticoagulant. To reduce donor-dependent variability, four units collected from donors of the same ABO blood group were pooled in a 2 L non-DEHP, DINCH-plasticized pooling bag (GCMS). Twenty pools (blood group: A = 7, B = 5, AB = 2, and O = 6) were prepared for this study. Before transferring the whole blood from the pooling bag to the individual triple bags, we poured the pre-existing CPDA-1 in the primary bag of the triple bag into one of the secondary satellite bags. We then disconnected the secondary bag to minimize the addition of excessive anticoagulant. From the 2 L pooling bag, 365 mL of anticoagulated whole blood was equally transferred to five different top-and-top blood bags: two non-DEHP prototype DINCH-plasticized triple blood bags manufactured by GCMS and Taechang (TC) Industry (Gongju-si, South Korea) and three DEHP-plasticized triple blood bags commercially available in our area, which were manufactured by GCMS, TC, and Fresenius Kabi (FK) (Bad Homburg, Germany). We produced RBC concentrates from each triple blood bag using the platelet-rich plasma method without leukoreduction. Homogenous quintets of matched RBC concentrates were produced within 4 h of collection and stored for 35 days in a standard blood bank refrigerator at 1–6°C. Aseptic techniques were used for all procedures, and connections between blood bags were made using a sterile connection device (Terumo Corporation, Tokyo, Japan).

Sampling and Sterility Testing

All units were sampled on days 1, 7, 14, 21, 28, and 35 of storage through sampling site couplers (4C2405, Fenwal Inc., Lake Zurich, IL, USA) to perform a panel of in vitro tests to analyze RBC concentrate quality. Blood (25 mL) was drawn using sterile syringes and 18 G needles following aseptic techniques. All RBC concentrate units were manually mixed gently and thoroughly before each sampling. At the end of 35 days of storage, each unit was tested for sterility using an automated microbial detection system (BacT/ALERT 3D, bioMérieux, Marcy l'Étoile, France) with both aerobe and anaerobe culture bottles.

RBC Concentrate Quality Analysis

RBC Characteristics

Cell counts, including total hemoglobin, hematocrit, and mean corpuscular volume (MCV), were assessed using an automated hematology analyzer (XN-9000, Sysmex, Kobe, Japan). The Fairbanks method was used to measure supernatant plasma hemoglobin using a spectrophotometer (DU 730, Beckman Coulter, Brea, CA, USA) [22]. Hemolysis was calculated according to the following formula [23]:

$$\text{Percent hemolysis (\%)} = (100 - \text{Hct}) \times \frac{\text{Supernatant hemoglobin (g/dL)}}{\text{Total hemoglobin (g/dL)}}$$

RBC Membrane Integrity

Eosin 5′ maleimide (EMA) binding test was performed to assess RBC membrane stability. In brief, after washing twice in phosphate-buffered saline, RBCs were incubated with EMA (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature in the dark. After staining, RBCs were washed twice with phosphate-buffered saline, and the final suspension was analyzed on a flow cytometer (FACSCanto II, BD, Franklin Lakes, NJ, USA). For each sample, 50,000 events were acquired, and RBCs were

gated by forward and side scatter parameters. The EMA binding was determined by calculating the ratio (%) of the mean fluorescence intensity of the test sample to that of a panel of six hematologically healthy normal controls.

RBC Metabolism

After extraction with 0.6 M perchloric acid and neutralization with 2.5 M potassium carbonate, 2,3-diphosphoglycerate (2,3-DPG) was measured using a commercialized kit (Roche, Basel, Switzerland). 2,3-DPG was measured only until day 21 as very low levels were expected on days 28 and 35 [14, 15]. Adenosine triphosphate (ATP) was measured from 12% (w/v) trichloroacetic acid extracts using ATP Hexokinase FS kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) on an automated immunoassay analyzer (ARCHITECT i2000SR Plus, Abbott Laboratories, Chicago, IL, USA). Glucose, lactate, K^+, and Na^+ were measured using an automated chemistry analyzer (Vitros 5,600, Ortho Clinical Diagnostics, Raritan, NJ, USA). The Cobas b 221 (Roche) blood gas analyzer was used to measure pH, pCO_2, and pO_2.

Plasticizer Analysis

DEHP and DINCH concentrations were analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (MS/MS). Samples were prepared by liquid-liquid extraction using n-hexane, evaporation in N_2 for 30 min, and reconstituted in 70% methanol with 0.1% formic acid. DEHP-d_4 was used as the internal standard. The reconstituted eluates were injected into the LC-30A Nexera (Shimadzu, Kyoto, Japan) ultra-high-performance liquid chromatography system equipped with the Synergi trap column (50.0 mm × 2 mm, 4 μm; Phenomenex, Torrance, CA, USA) and BEH column (50.0 mm × 2.1 mm, 1.7 μm; Waters, Watford, UK). The mobile phases were 0.1% formic acid in distilled water and 0.1% formic acid in methanol. The total run time was 12 min. We used the AB Sciex API 6500 (AB Sciex LLC, Framingham, MA, USA) triple quadrupole MS/MS system in multiple reaction monitoring mode for quantitation. Plasticizer concentrations were measured in a subset of the donors, 2 L pooled blood bags during component production, and individual RBC concentrate units throughout the storage period.

Statistical Analysis

Statistical analysis was conducted with receiving consultation from the Medical Research Collaborating Center at Seoul National University Hospital Biomedical Research Institute. Each DINCH bag (DINCH-GCMS, DINCH-TC) was compared with each of the three DEHP bags (DEHP-FK, DEHP-GCMS, and DEHP-TC) and also with the other DINCH bag. Nonparametrical-matched analysis using the Wilcoxon signed-rank test was performed using IBM SPSS Statistics 25 (Armonk, NY, USA). A p value of <0.05 was considered significant.

Results

Component Integrity

Tube seals and sampling site coupler connections for all units showed no leakage and functioned adequately throughout the storage period. There were no visual or physical differences between the DEHP and DINCH blood bags. All units tested negative for aerobic and anaerobic bacterial culture at the end of storage.

Hemolysis and RBC Characteristics

The results of the in vitro tests and statistical analysis are summarized in Table 1 and online supplementary Tables S1 and S2 (for all online suppl. material, see https://doi.org/10.1159/000535625). On day 35, the hemolysis rates in all bags of each study arm were below 0.8% (Fig. 2a). Both the DINCH bags showed higher hemolysis than the DEHP-GCMS and DEHP-TC bags starting as early as day 7 and DEHP-FK bag starting from day 14 (online suppl. Table S1). Although the median hemolysis in the DINCH-TC bag on day 35 was higher than in the DINCH-GCMS bag, the difference was not significant (Table 1). The MCV increased during the storage period in all the study arms, indicating RBC swelling (Fig. 2b). The MCV for both the DINCH bags was higher than the DEHP-FK bag starting from day 7 and DEHP-GCMS and DEHP-TC bags starting from day 14 (online suppl. Table S1). The EMA binding gradually decreased over the storage period in all study arms (Fig. 2c). The DINCH bags generally showed lower levels of EMA binding than the DEHP bags, and on day 35, the DINCH-TC bag showed lower EMA binding than all other bags (Table 1).

RBC Metabolism and Other Chemistry Tests

2,3-DPG levels decreased during the storage period, and the results were generally comparable between the study arms (Fig. 3a). The DEHP-FK bag showed a higher level of 2,3-DPG than the DINCH-GSMC bag on days 1 and 7 and the DINCH-TC bag on days 7 and 21 (Table 1, online suppl. Table S1, S2), but the differences were small. On day 21, 2,3-DPG was nearly depleted in all bags. ATP levels slowly decreased during the storage period (Fig. 3b). The DEHP-FK bag showed a slightly lower level of ATP than the DINCH bags on days 7, 21, 28, and 35 (Table 1, online suppl. Table S1, S2). All bags showed decreased glucose and Na^+ levels and increased lactate and K^+ levels throughout the storage period (Table 1, online suppl. Table S1, S2). The DEHP-FK bag exhibited lower glucose and higher lactate levels than the DINCH bags (Fig. 3c, d). Nevertheless, the rates of glucose consumption and lactate production were similar. Starting from day 7 onward, the DEHP-FK bag also demonstrated increased levels of K^+ and decreased levels of Na^+ compared to the two DINCH bags (Table 1, online suppl. Table S1, S2). K^+ and Na^+ levels were comparable between the DINCH bags and the DEHP-GCMS and DEHP-TC bags.

pH decreased in all bags throughout the storage period, and the results were generally comparable between the study arms (Fig. 4a). The DINCH bags had better gas permeability than DEHP bags. While all bags showed an increase in pCO_2 until day 14 followed by a decrease, the decrease was more significant in the DINCH bags than the DEHP bags (Fig. 4b). Similarly, all bags showed an increase in pO_2 throughout the storage period, and the increase was more significant in the DINCH bags than the

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Table 1. Analysis results for matched quintets (n = 20) of RBC concentrates stored in DEHP and DINCH bags with CPDA-1 on days 1 and 35[a]

Parameter	DEHP-FK	DEHP-GCMS	DEHP-TC	DINCH-GCMS	DINCH-TC
Day 1					
Hemolysis, %	0.023 (0.020–0.057)	0.029 (0.016–0.070)	0.021 (0.015–0.050)	0.024 (0.016–0.035)	0.021 (0.017–0.040)
Mean corpuscular volume, fL	90.8 (88.9–92.8)	91.0 (89.4–93.0)	91.3 (89.4–93.0)	91.1 (89.4–92.8)	91.3 (89.4–93.2)[b]
Eosin-5′-maleimide binding, %	97.9 (95.9–100.4)	98.2 (95.9–99.8)	98.4 (97.0–102.4)	98.4 (97.2–100.0)	97.8 (96.0–100.8)[d]
2,3-diphosphoglycerate, μmol/g Hb	11.6 (10.2–13.8)	10.9 (9.6–12.7)	11.2 (9.4–12.4)	10.8 (9.7–12.2)[b]	11.3 (9.3–12.2)
Adenosine triphosphate, μmol/g Hb	2.17 (2.03–2.36)	2.21 (2.07–2.33)	2.20 (2.13–2.32)	2.18 (2.05–2.36)	2.15 (2.11–2.34)
pH (37°C)	6.98 (6.96–6.98)	6.94 (6.93–6.96)	6.95 (6.93–6.96)	6.94 (6.93–6.96)[b]	6.94 (6.93–6.96)[b]
pCO_2, mm Hg	92.5 (86.0–93.9)	97.3 (91.0–101.1)	95.8 (89.3–99.4)	94.8 (88.3–98.3)[b,c]	95.8 (90.6–99.3)[b,c]
pO_2, mm Hg	59.6 (52.1–62.9)	59.2 (52.5–64.5)	59.1 (52.5–64.9)	58.0 (50.9–65.4)[d,e]	61.6 (53.9–69.8)[c,d,f]
Glucose, mg/dL	454 (445–473)	490 (468–504)	495 (484–508)	494 (483–513)[b,c]	493 (483–503)[b]
Lactate, mmol/L	5.05 (4.88–6.25)	5.21 (4.52–6.37)	4.81 (4.45–5.77)	4.70 (4.34–6.22)[b,c]	4.85 (4.38–5.75)[b,c]
K^+, mmol/L	6.4 (5.7–7.2)	6.2 (5.6–7.5)	6.1 (5.5–6.9)	6.1 (5.3–8.9)	6.0 (5.5–8.2)
Na^+, mmol/L	156 (153–157)	156 (154–158)	157 (155–158)	156 (154–158)	156 (153–158)
DEHP, mg/L[g]	4.6 (1.9–6.4)	3.9 (2.0–5.2)	4.1 (3.1–6.4)	1.3 (0.2–2.3)[b,c,d]	0.6 (0.2–1.4)[b,c,d]
DINCH, mg/L[g]	0.00 (0.00–0.03)	0.00 (0.00–0.03)	0.00 (0.00–0.01)	0.07 (0.02–0.14)[b,c,d]	0.05 (0.01–0.21)[b,c,d]
Day 35					
Hemolysis, %	0.204 (0.127–0.362)	0.240 (0.138–0.326)	0.222 (0.147–0.310)	0.297 (0.212–0.429)[b,c,d]	0.342 (0.230–0.493)[b,c,d]
Mean corpuscular volume, fL	98.7 (98.1–101.0)	98.4 (97.9–100.9)	98.3 (97.7–100.5)	99.4 (98.7–101.4)[b,c,d]	99.4 (98.7–101.5)[b,c,d]
Eosin-5′-maleimide binding, %	94.0 (91.7–97.3)	93.1 (91.0–98.1)	93.7 (90.4–96.6)	93.5 (91.1–97.3)[c,e]	92.6 (89.9–95.6)[b,c,d,f]
2,3-diphosphoglycerate, μmol/g Hb	Not tested	Not tested	Not tested	Not tested	Not tested
Adenosine triphosphate, μmol/g Hb	1.22 (1.10–1.28)	1.24 (1.16–1.34)	1.25 (1.17–1.42)	1.26 (1.15–1.38)[b]	1.26 (1.18–1.39)[b]
pH (37°C)	6.48 (6.45–6.50)	6.47 (6.43–6.48)	6.48 (6.44–6.49)	6.48 (6.45–6.50)	6.49 (6.46–6.50)[b,c,d]
pCO_2, mm Hg	118.4 (102.7–126.4)	123.0 (106.3–126.0)	122.6 (107.4–127.8)	90.3 (75.8–98.5)[b,c,d]	85.5 (77.2–99.9)[b,c,d]
pO_2, mm Hg	172.5 (145.4–221.7)	184.7 (119.2–210.2)	186.2 (160.8–224.4)	232.8 (220.7–255.9)[b,c,d]	234.8 (226.9–245.1)[b,c,d]
Glucose, mg/dL	137 (111–159)	170 (151–200)	184 (159–210)	179 (148–214)[b]	177 (151–197)[b,d]
Lactate, mmol/L	37.3 (34.6–40.0)	35.8 (32.8–38.0)	35.8 (32.9–37.9)	35.4 (33.3–38.8)[b,d]	36.3 (34.4–39.1)[c,d]
K^+, mmol/L	54.0 (48.8–56.3)	49.4 (48.0–53.0)	51.0 (47.4–52.8)	49.1 (44.7–52.7)[b,d]	50.0 (46.4–52.6)[b]
Na^+, mmol/L	135 (133–140)	138 (136–141)	138 (135–141)	139 (137–142)[b,c,d]	138 (136–142)[b,d]
DEHP, mg/L[h]	58.9 (37.3–64.0)	37.1 (29.1–58.3)	56.2 (44.6–70.1)	0.9 (0.5–1.3)[b,c,d]	0.7 (0.5–1.2)[b,c,d]
DINCH, mg/L[h]	0.00 (0.00–0.04)	0.01 (0.00–0.26)	0.00 (0.00–0.13)	0.89 (0.68–1.65)[b,c,d]	1.22 (0.56–1.58)[b,c,d]

FK, Fresenius Kabi; GCMS, Green Cross Medical Science; TC, Taechang. [a]Data are presented as median (Q_1-Q_3). [b]$p < 0.05$ compared to storage in DEHP-FK. [c]$p < 0.05$ compared to storage in DEHP-GCMS. [d]$p < 0.05$ compared to storage in DEHP-TC. [e]$p < 0.05$ compared to storage in DINCH-TC. [f]$p < 0.05$ compared to storage in DINCH-GCMS. [g]n = 11. [h]n = 12.

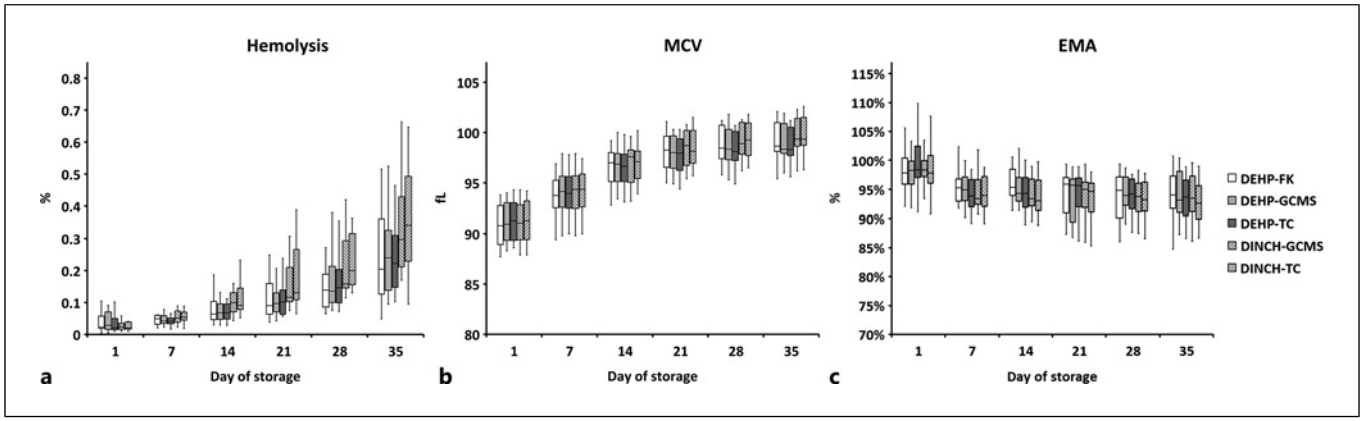

Fig. 2. Box and whisker plots of hemolysis and RBC characteristics of RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20). The DINCH bags exhibited increased hemolysis (**a**), higher MCV (**b**), and reduced EMA binding (**c**) compared to the DEHP bags. On day 35, all bags in each study arm displayed hemolysis rates of <0.8% (**a**). Significance was analyzed using the Wilcoxon signed-rank test (Table 1, online suppl. Tables S1, S2).

DEHP bags (Fig. 4c). On day 35, there was no significant difference in the pCO_2 and pO_2 levels between the two DINCH bags (Table 1).

Plasticizer Levels

The median (Q_1–Q_3) levels of DEHP and DINCH in the donors' blood samples (n = 40) were 0.57 (0.28–0.89) and 0.00 (0.00–0.00) mg/L, respectively. The median (Q_1–Q_3) levels of DEHP (mg/L) and DINCH (mg/L) in the pooled blood (n = 10) during component production were 0.30 (0.24–1.50) and 0.01 (0.00–0.02), respectively. The DEHP levels gradually increased throughout the storage period in the DEHP bags, while the remaining were nearly undetectable in the DINCH bags (Fig. 5a). The DEHP bags showed significantly higher DEHP levels than the DINCH bags from day 1 onward (Table 1, S1, and S2). Conversely, the DINCH levels gradually increased throughout the storage period in the DINCH bags, while the remaining were nearly undetectable in the DEHP bags (Fig. 5b). The DINCH bags showed significantly higher DINCH levels than the DEHP bags from day 1 onward (Table 1, online suppl. Table S1, S2). Compared to DEHP, DINCH exhibited considerably lower levels of plasticizer leaching into blood bags.

Discussion

Plasticizers can leach out from the material to its content or surroundings because they are not covalently bound to the products [8, 24]. Hence, plasticizers accumulate in our environment and the ecosystem, making them a ubiquitous material detected in aquatic systems, drinking water, and soil [25]. Because of this characteristic, humans and animals can be easily exposed to plasticizers through various routes, such as inhalation, ingestion, and skin contact. For this reason, there has been growing concern over the safety of certain plasticizers, particularly phthalates, which have been linked to health issues. The biological harmfulness of DEHP, the most widely used phthalate, has been mainly observed in reproductive and developmental toxicity through animal models [7, 8, 26]. Numerous studies have attempted to determine the direct human toxicity of DEHP in various fields such as testosterone production, hypospadias, cryptorchidism, infant and adolescent growth, and decreased anogenital distance [27]. However, the conclusions regarding DEHP's human toxicity have not been clear or consistent.

DINCH, along with BTHC, DEHT, DOTH, and DL9TH, is being investigated as a potential nontoxic substitute for DEHP in blood bags used for RBC storage [9, 14–20]. Although data from human studies are limited, DINCH has shown low or no relationship to toxicity, genotoxicity, carcinogenicity, or toxicity to reproduction in animal models [28–31]. DINCH has been approved by the European Union for food packaging and has been widely used in toys and childcare products [31, 32]. DINCH-PVC showed similar viscosity and mechanical properties to DEHP-PVC and high resistance to degradation to steam sterilization, allowing the potential to be a promising alternative to DEHP for medical device industrialization [33, 34].

This study evaluated DINCH as an alternative plasticizer to replace DEHP in blood bags for RBC storage with CDPA-1 anticoagulant. We used a pool-and-split study design to obtain homogeneously matched quintets of adult-sized RBC concentrates for comparison. Some previous studies have used pediatric-sized units [15, 16], which may have different volume-to-area properties of

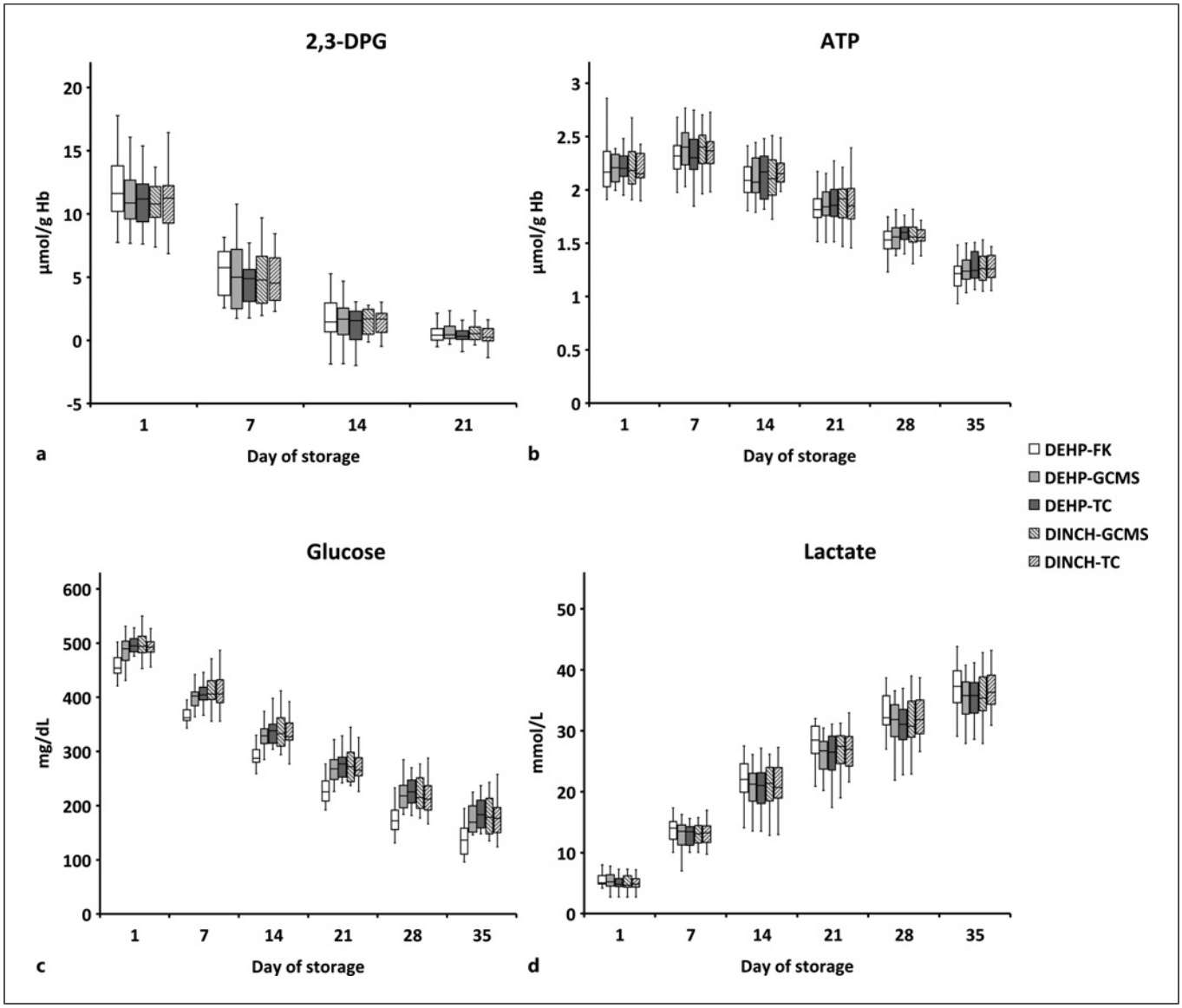

Fig. 3. Box and whisker plots of 2,3-DPG, ATP, glucose, and lactate levels in RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days ($n = 20$). The DINCH and DEHP bags exhibited comparable levels of 2,3-DPG (**a**) and ATP (**b**) throughout the storage period. The DEHP-FK bag showed a decrease in glucose levels (**c**) and an increase in lactate levels (**d**) compared to the other bags. However, the rates of glucose consumption (**c**) and lactate production (**d**) were similar in the different study arms. Significance was analyzed using the Wilcoxon signed-rank test (Table 1, online suppl. Tables S1, S2).

the bags and concentrations of the plasticizer leaching into the blood compared with adult-sized bags. Because DEHP can easily migrate from the tubing and bags into the blood, we used DEHP-free prototype collection systems and pooling bags made with DINCH to eliminate DEHP contamination during blood collection and RBC concentrate production. Although there was a possibility of DINCH cross-contamination into the DEHP study arms, we prioritized preventing DEHP cross-contamination due to its well-known protective effect on RBC stability, a limitation noted in some previous studies [9, 15, 16, 18]. Additionally, we assumed that the levels of DINCH in the DEHP study arms would be very low because RBC concentrates were produced within 4 h of blood collection, and DINCH leaches into the blood at a much slower rate than DEHP [17]. As expected, DINCH was not detected in most of the donor samples and remained at nearly undetectable levels throughout the storage period in DEHP bags. Therefore, the levels of DINCH would have a negligible impact on the hemolysis results of the DEHP bags.

The RBC concentrate units in this study were not leukoreduced and were stored using CDPA-1 as the anticoagulant with no other additive solutions. Previous studies on DEHP alternatives were mainly based on leukoreduced components [9, 14–19], and only one pilot

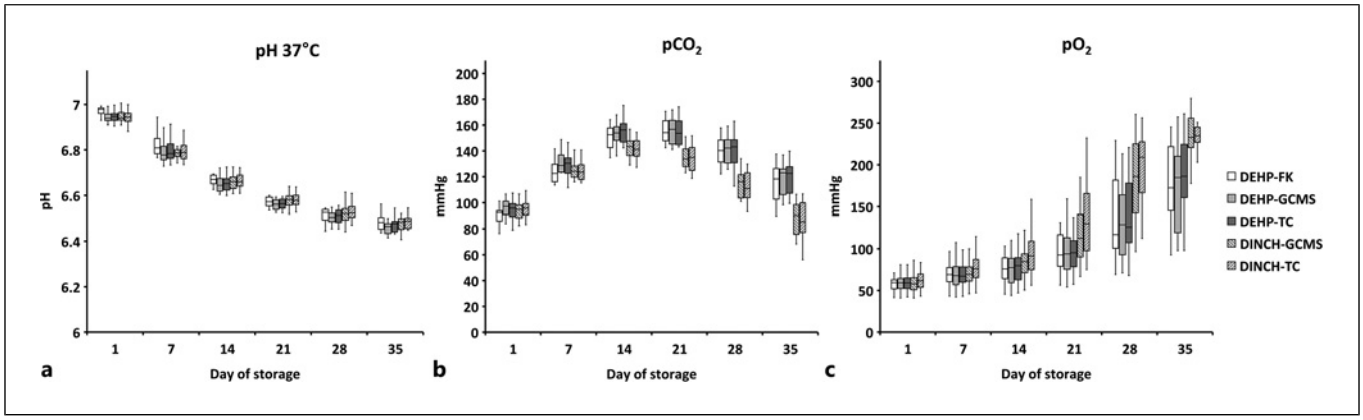

Fig. 4. Box and whisker plots of pH, pCO_2, and pO_2 levels in RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days (n = 20). pH levels were comparable between the DINCH and DEHP bags (**a**). DINCH bags demonstrated enhanced gas permeability, as evidenced by decreased pCO_2 (**b**) and increased O_2 (**c**) levels compared with DEHP bags. Significance was analyzed using the Wilcoxon signed-rank test (Table 1, online suppl. Tables S1, S2).

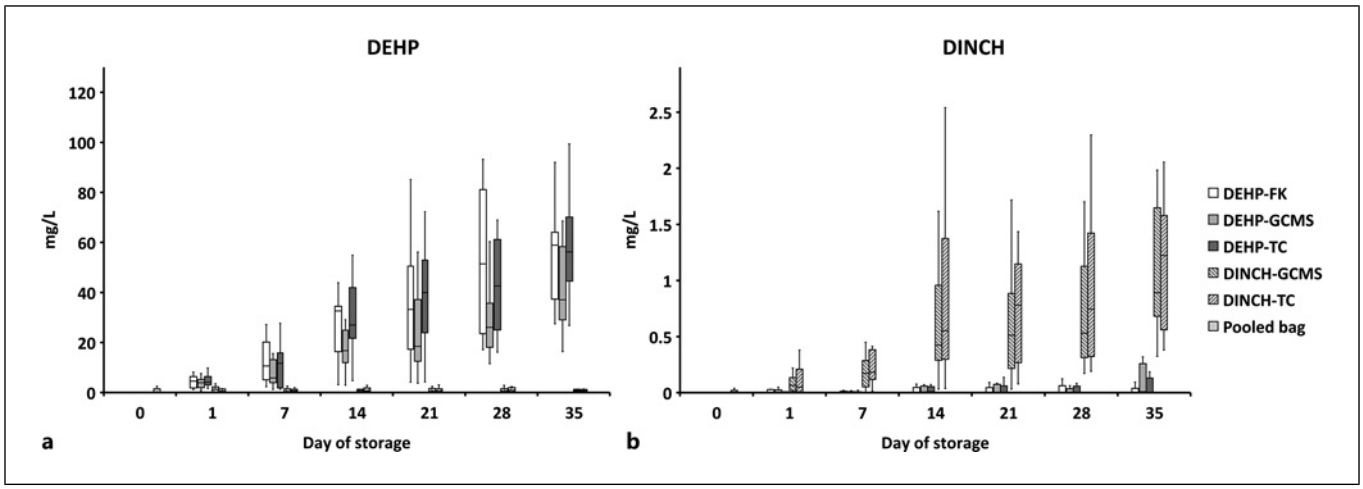

Fig. 5. Box and whisker plots of DEHP (**a**) and DINCH (**b**) levels in pooled blood (day 0) and RBC concentrate units stored in DEHP- and DINCH-plasticized blood bags for 35 days. DINCH exhibited considerably lower levels of plasticizer leaching into blood bags compared to DEHP. Significance was analyzed using the Wilcoxon signed-rank test (Table 1, online suppl. Tables S1, S2).

study has been reported for nonleukoreduced RBC concentrates [20]. Prestorage leukoreduction has been reported to have various beneficial effects on RBC storage quality, including hemolysis [35–37]. Without a direct comparison, it is unclear if leukoreduction may improve RBC concentrate properties during storage in DINCH bags. However, universal leukoreduction of blood components and routine use of additive solutions are not available in every jurisdiction or country. Other than several high-income countries in Europe, universal leukoreduction has been adopted in only a few countries, including Canada, Japan, the United Arab Emirates, and Qatar [38, 39]. In the USA, over 95% of the RBC/whole blood units and platelet units are leukoreduced [40]. In South Korea, leukoreduction for RBC and platelet components is selectively performed for high-risk patients [41], and SAGM is used only when producing prestorage-leukoreduced RBC concentrates. According to an international survey of low- and middle-income countries, leukoreduction was available in only 51.9% of the respondents [21]. However, people living in low- and middle-income countries constitute 84% of the world's population [42]. Since most previous studies have employed leukoreduction and additive solutions, our study's results will hold value to the majority of areas where blood product manufacturing follows conventional methods with limited utilization of these options.

The main issue when searching for a viable surrogate to replace DEHP in RBC storage bags is concerned with DEHP's protective effects on RBC stability. Alternative plasticizers should not only have a nontoxic biosafety profile but also demonstrate sufficient ability to preserve RBCs. The US FDA requires less than 1% of hemolysis at the end of RBC storage [43], while the Council of Europe Guideline has a more stringent requirement of 0.8% [44]. In this study, we found that the median hemolysis rates were higher in the DINCH bags than in the DEHP bags. Moreover, the difference between them gradually increased throughout the storage period. However, the hemolysis rates for all individual DINCH bags were well within the acceptable regulatory limits, demonstrating their potential as a feasible alternative to DEHP bags. Other previous studies on DINCH bags have similarly shown comparable or slightly increased levels of hemolysis compared with DEHP bags in various experimental settings [15–18].

Two interesting hemolysis-related factors assessed in these studies on DINCH bags were (1) mixing the RBC concentrate units during storage and (2) the additive solution used for RBC preservation. A study on DINCH bags with leukoreduced RBC concentrates in Optisol (AS-5) reported better RBC characteristics, including hemolysis, when mixed weekly compared to being statically stored for 42 days [18]. The positive effect of mixing was presumed to be due to enhanced plasticizer migration or redistribution of component constituents [17, 18]. In this study, both the DINCH and DEHP bags were mixed weekly before sampling. The additive solution used could also remarkably affect hemolysis in the DINCH bags. A study on DINCH bags with leukoreduced RBC concentrates and four different additive solutions (SAGM, PAGGSM, PAGGGM, and AS-3) reported increased hemolysis and MCV levels only in RBCs stored with SAGM when compared to the conventional DEHP/SAGM combination [17]. Additionally, compared to SAGM, PAGGSM has exhibited reduced microvesiculation and higher osmotic stability for RBC storage [45]. These findings suggest that while DINCH may be inherently inferior to DEHP in terms of preserving RBC stability, periodic mixing of RBC concentrate units during storage and/or using next-generation additive solutions with improved function may help compensate for this disadvantage.

In addition to the findings on hemolysis, DINCH bags also showed differences in MCV and EMA binding in this study. RBCs stored in DINCH bags with CPDA-1 showed a slight increase in MCV compared to DEHP bags. Increased MCV indicating RBC swelling is commonly observed during RBC storage [46]. Based on the type of plasticizer and additive solution used, varying levels of MCV increase have been reported in previous studies [9, 14–19]. The EMA binding test is used to diagnose hereditary spherocytosis, characterized by a deficiency in RBC membrane proteins leading to detachment of the lipid bilayer from the cytoskeleton [47]. The intensity of fluorescence signal derived from EMA bound to RBC membrane proteins measured by flow cytometry is an indicator of the RBC membrane integrity, with an estimated cutoff value for EMA binding in hereditary spherocytosis to be <86.9% [48]. In this study, EMA binding decreased in all study arms throughout the storage period, but the values mainly remained above 90%. The DINCH bags showed a small yet significant decrease in EMA binding compared to the DEHP bags on day 35. Another parameter used to measure the loss of RBC membrane integrity is the RBC microvesicle (microparticle). DEHP inhibits irreversible loss of the RBC membrane caused by microvesicle formation which can lead to increased osmotic fragility and hemolysis [5, 17]. Previous studies comparing DINCH or DEHT bags to DEHP bags have reported an increase in microvesicle count throughout storage, consistent with an increase in hemolysis [14–16]. The EMA binding and microvesicle counts are based on opposing effects on the RBC membrane. This relationship is well demonstrated in Figure 2, which shows an inverse relationship between hemolysis and EMA binding. Compared to the DEHP bags, the DINCH bags exhibited increased hemolysis and decreased EMA binding, while the DINCH-TC and DEHP-FK bags generally showed the corresponding maximum or minimum median values.

In this study, the DINCH and DEHP bags showed no remarkable differences in the levels of 2,3-DPG, ATP, and pH. However, the DINCH bags exhibited higher pO_2 and lower pCO_2 levels compared to the DEHP bags. There was no correlation between the pH values and blood gas partial pressures, suggesting that the changes in pO_2 and pCO_2 are attributed to the gas permeability properties of the bags rather than the metabolic activities of RBCs. Similar findings have been reported in previous studies [14, 15]. DEHP-PVC has been reported to have low permeability to O_2 and CO_2, limiting its suitability for platelet storage [49]. PVC containers plasticized with BTHC or trioctyl trimellitate are nowadays preferred for platelet storage due to their higher gas permeability, allowing for longer platelet storage compared to DEHP-PVC containers [2]. It remains unclear whether this increased gas permeability of DINCH has any clinical impact on RBC transfusion for patients.

While the DEHP-FK bag showed decreased glucose and slightly increased lactate levels, the other four had comparable levels of these metabolites. The noticeable gap in the glucose level observed as early as day 1 in the DEHP-FK bag might be due to differences in the composition of CPDA-1 from different manufacturers. Although we removed excess CPDA-1 from the primary bag

of the triple bags before distributing the whole blood from the pooling bag during the production process, some remnant CPDA-1 may have affected the results. However, as shown in Figure 3, the five study arms had comparable rates of glucose consumption and lactate production. The relationship between hemolysis and K^+ level in the blood bags is another interesting issue. Hemolysis in blood samples is known to increase K^+ and decrease Na^+ concentrations in the plasma [50, 51]. In this study, although the DEHP-FK bag showed the lowest level of hemolysis, it had the highest K^+ and the lowest Na^+ levels. This lack of correlation between hemolysis and K^+ levels has also been observed in previous studies on the DINCH and DEHT bags [9, 14, 15, 17, 18]. The reason for this unexpected yet repetitive finding is unclear, and further research is necessary to better understand this phenomenon.

While baseline DEHP levels were detected in the donors' blood samples at low concentrations, indicating its ubiquitous presence as an environmental contaminant, DINCH was not detected. DINCH concentrations also remained at nearly undetectable levels in the DEHP bags throughout the storage period. The levels of DINCH in the DINCH bags on day 35 in this study were lower but within a comparable range to the results observed on day 42 in previous studies [17, 19]. The DEHP concentrations in the DINCH bags remained consistent with the donors' baseline levels throughout the storage period. The median DEHP levels of the three different DEHP bags on day 35 in this study increased to a range of 37.1–58.9 mg/L, which is comparable to the results observed on days 20 and 42 in previous studies [9, 17, 19, 52]. DEHP-FK and DEHP-TC bags exhibited higher levels of DEHP leaching than the DEHP-GCMS bag, possibly due to variances in the plasticizer content among manufacturers. Variations in DEHP and DINCH levels among studies can be attributed to differences in analytical methods and chemical composition of the blood bags.

This study has some limitations. First, the magnitude of the differences in many of the test results, even when statistically significant, was not considerably large. It is unclear whether they are clinically relevant. Second, the gradual reduction in the RBC concentrate unit volume is another factor to consider. Because we took considerable amounts of samples from the units for testing every week, the volume of blood where the plasticizer is being accumulated decreased throughout the storage period. A simple comparison of days 1 and 35 might have produced more straightforward results. Third, although the cross-contamination of DINCH into the DEHP study arms was very low, its impact cannot be ruled out. Finally, other differences, such as the collection volume, time delay between collection and processing, leukoreduction, irradiation, washing, centrifugal force, centrifugation time, and production method (buffy coat vs. platelet-rich plasma), can all contribute to variability in results.

In conclusion, this study demonstrates that non-leukoreduced RBC concentrates stored for 35 days in DINCH-plasticized blood bags with CDPA-1 exhibit comparable quality to those stored in DEHP bags. While RBCs in DINCH bags showed slightly higher hemolysis than those in DEHP bags, all hemolysis levels remained below the current regulatory limits. Our findings suggest that DINCH is a promising alternative to DEHP in blood bags for RBC storage, even without the use of leukoreduction and next-generation additive solutions. The results of our study hold significance for regions with limited resources in blood product manufacturing. To gain a more comprehensive understanding of this issue and develop a suitable solution, further accumulation of information on the validation of alternative plasticizers, in conjunction with various factors related to RBC concentrates, including additive solutions, storage conditions, and component production methods, is required.

Statement of Ethics

This study was ethically approved by the Institutional Review Board of Seoul National University Bundang Hospital (B-1705-395-309, B-1707-406-301) and performed in accordance with the Declaration of Helsinki. All study participants provided written informed consent to participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Kim H. collected and summarized the data and wrote the manuscript. Lee K., Seo S.H., Hong Y.J., Hwang S.M., Park J.S., and Song J. critically revised and supported the study. Park K.U. supervised the study and edited the manuscript. All authors have reviewed and approved the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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