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Additive solution-7 reduces the red blood cell cold storage lesion

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BACKGROUND: Transfusion of long-stored red blood cells (RBCs) is associated with decreased in vivo RBC recovery, delivery of RBC breakdown products, and increased morbidity and mortality. Reducing the burden of this RBC “storage lesion” is a major challenge in transfusion medicine. Additive solution-7 (AS-7) is a new RBC storage solution designed to improve RBC metabolism by providing phosphate and increasing buffering capacity.

STUDY DESIGN AND METHODS: Storage quality in AS-7 was measured in a prospective, randomized, three-center trial using units of whole blood from healthy human subjects whose RBCs were stored for up to 56 days in AS-7 ($n = 120$) or for 42 days in the control solution AS-1 ($n = 60$).

RESULTS: Hemolysis and shedding of protein-containing microvesicles were significantly reduced in RBCs stored in AS-7 for 42 and 56 days compared with RBCs stored in AS-1. Autologous in vivo recoveries of RBCs stored in AS-7 was $88 \pm 5\%$ at 42 days ($n = 27$) and $82 \pm 3\%$ at 56 days ($n = 27$), exceeding recoveries of RBCs stored in currently used solutions.

CONCLUSION: Increasing the phosphate, pH range, and buffer capacity of a RBC storage system allowed RBCs to be stored better and longer than currently approved storage systems. AS-7 ameliorates the long-term storage lesion resulting in significantly increased viability in vitro and in vivo.

Red blood cells (RBCs) are the most commonly transfused blood products.¹ However, the occurrence of antigenic blood groups on RBCs means that large numbers of RBC units are required in the inventories of regional blood systems and hospitals to assure that needed types are readily available.^{2,3}

RBCs for transfusion are prepared either from whole blood collected into CPD or CP2D or from apheresis collection into CP2D or ACD-A. After being processed into components, additional nutrients are provided to the RBCs for long-term storage in the form of an additive solution (AS).⁴ RBCs for transfusion may also be leukoreduced before storage. These ASs were developed to provide volume for the dilution of metabolic waste; nutrients, especially adenine; and membrane protectant sugars. The current AS allow RBCs to be stored for up to 42 days with a mean in vivo recovery at the end of storage of $82 \pm 7\%$

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(n = 641), 12% of samples showing less than 75% recovery, and hemolysis of 0.4% (n = 14,087) or 0.5% (n = 344, unpublished).⁶ These performance characteristics suggest that ASs are effective and safe, and three ASs are licensed and in use in the United States, AS-1, AS-3, and AS-5, which perform equivalently.⁷⁻⁹ Additional ASs have been approved for use in other countries including SAG-M, MAP, and PAGGSM for storage for 35 to 42 days.¹⁰⁻¹² The use of 42-day AS-stored RBC products has allowed the development of highly efficient national blood programs with approximately 95% of donated RBCs finding a recipient in Western countries.

Recent studies have raised concerns about the safety of stored RBCs associated with the development of a clinically relevant RBC storage lesion, which include increased morbidity and mortality in a number of retrospective clinical trials.¹³⁻¹⁹ Recently, the release of iron from stored and transfused RBCs has been shown to cause inflammation and increased mortality in animal models of transfusion.²⁰ Cumulative evidence that long storage results in increased frequency and severity of transfusion-related lung injury, and the realization that some donors' cells store poorly in present AS systems,^{6,16,21} have brought attention to the need to reduce the RBC storage lesion while maintaining RBC availability and minimizing costs.

AS-7 (SOLX) is a new alkaline RBC storage solution containing bicarbonate, adenine, glucose, mannitol, and phosphate designed to improve RBC metabolism during storage by increasing the range and capacity of pH buffering. It was previously known in the literature as EAS-81 (experimental AS) experimental AS-81.²² This article describes the storage of RBCs in AS-7 for 42 and 56 days within a range of conventional processing times (<2 and 6-8 hr at room temperature), using blood banking systems and under current regulations for clinical transfusion. These data suggest that this new storage system significantly reduces the storage lesion as assessed by improved biochemical status, decreased vesicle formation, reduced hemolysis, and increased in vivo recovery at the end of conventional and prolonged periods of storage. These results have led to the Food and Drug Administration (FDA) approval of AS-7, the first new AS approved in the United States in 25 years. In Europe, a "CE" mark has been granted for 8-week storage.

MATERIALS AND METHODS

Clinical trial design

We conducted a three-center, three-arm-evaluation of the AS-7 (LEUKOSEP HWB-600-XL leukoreduction filtration system with CPD and SOLX AS for whole blood, test, Hemerus Medical, LLC, St Paul, MN; Table 1) compared to AS-1 (RZ2000 leukoreduction filter with CPD anticoagulant and Adsol AS, control, 4R3335E, Fenwal, Inc., Lake Zurich, IL), an FDA-approved system for the collection,

TABLE 1. Composition and chemical characteristics of AS-1 and AS-7

Constituents (mmol/L)	AS-1, Adsol	AS-7, SOLX
NaCl	154	
NaHCO ₃		26
Na ₂ HPO ₄		12
Adenine	2	2
Glucose	111	80
Mannitol	41	55
pH	4.6-7.2 ²³	8.5
Density (g/mL)	1.012	1.01
Osmolality (mOsm/L)	462	237

filtration, and component processing of whole blood (Table 1). The final composition of AS-7 is reached after addition of the acidic glucose component, which is part of the collection set but only added during the RBC processing phase. Healthy human subjects that met AABB and FDA criteria for voluntary blood donation were enrolled under a protocol approved by the FDA, the US Army's Human Subjects Research Protection Office, and the institutional review boards of the three research sites. Volunteers were enrolled without targeted recruitment or exclusion due to previous analyses of hemolysis or in vivo viability data. The study conforms to guidelines of the Declaration of Helsinki. Subjects were randomly assigned to have their whole blood collected and processed into RBC and plasma components stored in one of three arms: 1) control processed within 8 hours and stored in AS-1 at 1 to 6°C for 42 days (n = 60), 2) test processed within 2 hours and stored in AS-7 at 1-6°C for up to 56 days (n = 60), and 3) test processed within 8 hours and stored in AS-7 at 1 to 6°C for up to 56 days (n = 60). The total number of collections for each arm was balanced over time but was not subject to formal randomization.

Briefly, 500 ± 50 mL of whole blood was collected from healthy research subjects (n = 180) into CPD using either a test or a control collection system. Whole blood was held at room temperature before filtration and component processing. Units were whole blood filtered at room temperature after either 2 hours (one test arm) or 6 hours (one test arm and control arm), centrifuged with a validated hard centrifugation protocol, and plasma was manually expressed. For long-term storage at 1 to 6°C, control RBCs were placed in AS-1 for 42-day storage, and test RBCs were placed in AS-7 for 42- or 56-day storage. All units were evaluated with a panel of in vitro assays at the beginning and end of storage. Only AS-7-stored RBCs were evaluated for in vivo autologous recoveries, n = 27 per arm (n = 14 for units processed in less than 2 hr and n = 13 for units processed in 6-8 hr).

RBC hematologic and chemical analysis

Timed samples were analyzed for pH at 37°C, free hemoglobin (Hb), adenosine 5'-triphosphate (ATP),

2,3-diphosphoglycerate, extracellular potassium, extracellular glucose, extracellular lactate, packed cell volume, and mean corpuscular Hb concentration (MCHC) as previously described.²⁴ The quantity of total protein shed in microvesicles was determined as described previously.²⁵ Each center conducted all assays within its own institution. A formal, a priori comparison of analytical methods was not conducted; however, no significant center effects were observed for the study outcomes (data not shown).

Chromium-51–labeled RBC 24-hour recovery

On Days 42 and 56, each unit was inspected for any signs of unusual hemolysis or discoloration indicative of bacterial growth. The unit was well mixed by hand (1 min), and approximately 15 mL of the RBCs removed and labeled with approximately 15 μ Ci of chromium-51 (⁵¹Cr) with standard techniques.²⁶ The labeling agent, ⁵¹Cr sodium chromate, was mixed aseptically with the RBCs at room temperature for 30 minutes. One double-volume saline wash was conducted. An aliquot of the final volume was reserved for assay as a standard, and the remaining labeled cells (approx. 10 mL) were injected into a free-flowing peripheral vein. Samples (5 mL each) were taken from a contralateral vein at time intervals within the first 30 minutes after infusion as well as at 24 hours through a butterfly needle. The samples were counted in a gamma counter to determine ⁵¹Cr activity. The activity of the samples from the first 30 minutes was back-extrapolated to determine a T₀ activity. The amount of RBCs and the amount of radioactivity infused were determined based on the methods of Moroff and coworkers²⁶ and the International Committee for Standardization in Hematology.²⁷ All ⁵¹Cr specimens used for survival analysis were counted at the same time and corrected for decay. Analysis of Day 56 RBC survival used modified elution rate calculations based on differential subtraction of decayed radioactivity from Day 42 RBC infusion.

Data handling, validation, and statistical analysis

Data were entered onto a preapproved microcomputer spreadsheet (Excel, Microsoft Corp., Redmond, WA) by investigators at the three sites and validated by auditors from Hemerus Medical, LLC. Data are presented as individual points, mean, and SD, and analysis uses t test analysis for independent or paired samples or analysis of variance with Bonferroni correction when more than two groups are compared.

RESULTS

A total of 180 units were successfully tested. For the 120 units collected into the test system, leukoreduction was accomplished in 21.2 ± 5.2 minutes with $93.7 \pm 1.4\%$ RBC

TABLE 2. Volume and Hb concentration ([Hb]) of leukoreduced, postprocessing stored units

Groups	Volume (mL)	[Hb] (g/dL)
AS-1, 6- to 8-hr processed	308 $\pm$ 17	19.6 $\pm$ 1.0
AS-7, <2-hr processed	311 $\pm$ 18	19.4 $\pm$ 1.1
AS-7, 6- to 8-hr processed	311 $\pm$ 17	19.0 $\pm$ 1.0

filtration mass recovery and a 4.3 ± 0.4 log reduction in white blood cell (WBC) content with a mean final WBC count of 2.5×10^5 WBCs per unit (median, 1.1×10^5 ; range, 2.6×10^4 – 2.6×10^6). Analysis of center effects showed no significant effects for the study outcomes and, therefore, all the results from the three centers were pooled and analyzed together.

Volume and Hb concentration of stored units is presented in Table 2. The extracellular pH of AS-7 RBC units at the beginning of their storage was 7.02 ± 0.04 and 6.96 ± 0.03 for less than 2 and 6- to 8-hour storage, respectively, which are significantly lower than the 7.06 ± 0.3 ($p < 0.01$) observed at the beginning of storage in AS-1 despite the higher pH of AS-7 (Table 1). Lactate concentrations of 1.8 ± 0.6 and 3.0 ± 0.7 mmol/L ($p < 0.01$) were observed in units processed in less than 2 hours or processed between 6 and 8 hours after collection, respectively. However, by Day 42 the AS-7 groups had a higher pH than AS-1–stored RBCs and were only slightly lower on Day 56 ($p < 0.01$, Fig. 1A). AS-7 units had a mean glucose consumption of 0.76 mmol/week (Fig. 1B) and a mean lactate production of 1.3 mmol/week as the concentration increased to 29 ± 5 mmol/L by Day 42 of storage (Fig. 1C). Lactate production dropped below the mean for the first 6 weeks of storage to approximately 0.48 mmol/week after Day 42 of storage. Storing equivalent amounts of RBCs in AS-1 in the control arm of sAZ the study was associated with 22% lower glucose consumption of 0.60 mmol/week (Fig. 1B) and lower lactate (Fig. 1C). AS-7 buffering capacity is bicarbonate based; as a result, the bicarbonate loss by Day 42 of storage in AS-7 was significantly greater compared to the same period of storage in AS-1 and achieved the same concentration as that of Day 42 AS-1–stored RBCs by Day 56 of storage in AS-7 solution (Fig. 1D).

These results suggested an increased glycolytic flux in well-buffered AS-7–stored RBCs compared to RBCs stored in AS-1. To confirm that the increased glycolytic flux resulted in increased energy production, we measured the intracellular levels of ATP. The intracellular ATP concentration of the overall AS-7–stored RBC groups ($n = 120$) declined from a mean of 4.3 ± 0.7 to 3.9 ± 0.7 μ mol/g Hb by Day 42. RBC ATP concentrations in AS-1–stored units ($n = 60$) decreased from 4.4 ± 0.7 to 3.6 ± 0.8 μ mol/L/g Hb during the same period (Fig. 1E). AS-7–stored RBC ATP levels declined to 3.1 ± 0.8 μ mol/g Hb by Day 56 of storage (Fig. 1E), well within the levels considered sufficient to maintain RBC viability.²⁸ As a result, the ATP

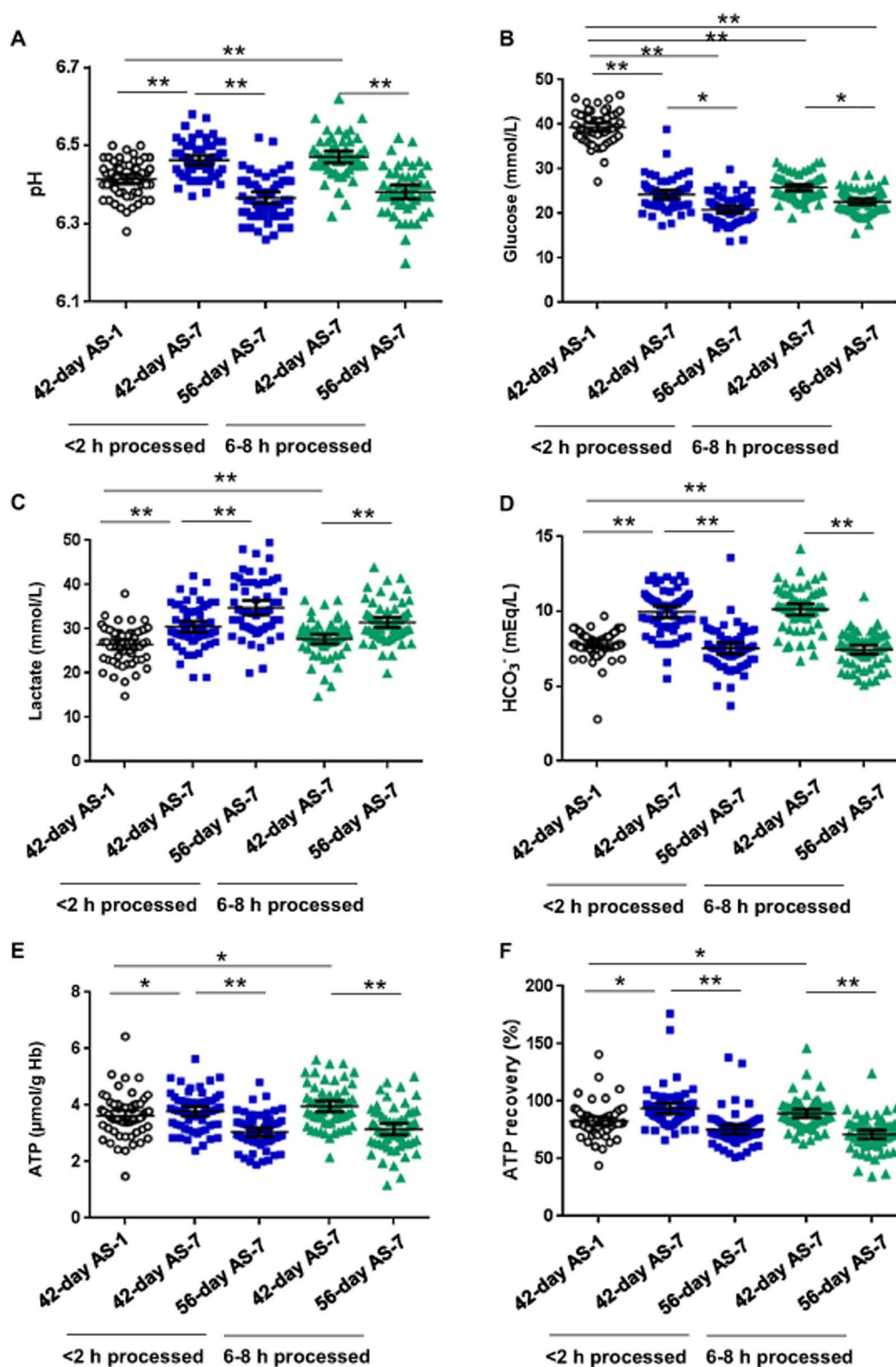


Fig. 1. Metabolic status of 42- or 56-day-stored RBCs in AS-1 or AS-7 processed in less than 2 hours or between 6 and 8 hours postcollection. (A) Extracellular $\text{pH}_{37^\circ\text{C}}$. (B) Glucose concentration. Day 0 concentrations of glucose in units stored in AS-1 and AS-7 are 52.3 and 41.5 mmol/L, respectively. (C) Lactate concentration. (D) Bicarbonate (HCO_3^-) concentration. (E) Intracellular ATP levels. (F) ATP recovery at the end of storage compared with immediately after processing. Individual data are presented along with mean $\pm$ 1 SD. n = 60 per group. *p < 0.05; **p < 0.01.

recoveries, assessed as the percentage of ATP level after storage of the prestorage level, were significantly higher in the AS-7 groups ($93.7 \pm 17.9\%$ and $89.5 \pm 13.7\%$ for less than 2 hr to processing and 6 to 8 hr to processing, respectively) than in the AS-1–stored RBC control processed between 6 and 8 hours postcollection ($82.5 \pm 14.6\%$, $p < 0.01$) by Day 42 of storage (Fig. 1F).

RBC storage lesion under standard conditions typically results in reduced integrity of the RBC membrane, which results in loss of biconcave shape, formation of echinocytes, shedding of protein-rich exocytic microvesicles, hemolysis, and decreased in vivo survival of circulating RBCs. We analyzed morphologic changes in RBCs and determined the level of protein-rich microvesicles shed by long-term–stored RBCs.²⁹ The morphology index (scale up to 100 in a perfect biconcave RBCs) of AS-7–stored RBCs for 42 days were 81 ± 8 and 80 ± 6.6 for units processed before 2 hours and within 6 to 8 hours postcollection, respectively, compared with 69 ± 8 for Day 42 AS-1–stored RBCs ($p < 0.01$). By Day 56, AS-7–stored RBCs maintained approximately 10% higher morphology indices (79 ± 7 and 77 ± 7 , for the same groups, respectively) than Day 42 AS-1–stored RBCs ($p < 0.05$; Fig. 2A). Exocytic microvesicle shedding is a result of storage, which further accentuates the RBC damage as a result of the loss of membrane and cytoplasmic proteins required for membrane deformability and is associated with morphologic changes in RBCs. We found that 56-day AS-7–stored RBCs contained approximately 40% less shed microvesicle protein than 42-day AS-1–stored RBCs (16.8 ± 11.3 mg protein/dL RBCs vs. 28.9 ± 18.2 mg protein/dL RBCs, respectively; $p < 0.001$, Fig. 2B). Interestingly, while AS-7 ameliorated the membrane lesion of stored RBCs, it had no effect on the leakage of potassium from RBCs (Fig. 2C), suggesting that the cation-exchange transporter function may not be affected by the changes associated with the AS-7 system AS at refrigerated storage temperatures. Finally, to determine whether AS-7 storage prevented the loss of RBC integrity ex vivo and in vivo, we first determined the hemolysis in AS-7 units at different time points, which averaged $0.29 \pm 0.11\%$ at 42 days with a maximum observed value of 0.72% and $0.42 \pm 0.17\%$ at 56 days, with all units having a hemolysis of less than 0.90% ($n = 120$). AS-1 control units processed within 8 hours of collection had hemolyzed fractions of $0.39 \pm 0.21\%$ (maximum, 0.98%), which was significantly higher than in the units stored in AS-7 at 42 days (Fig. 2D). Second, we measured the 24-hour recoveries of ^{51}Cr -labeled RBCs stored in AS-7 as an assessment of storage lesion resulting in early in vivo death. Recovery averaged $88 \pm 5\%$ ($n = 27$) at 42 days and $82 \pm 3\%$ ($n = 27$) at 56 days of storage (Fig. 2E). All units stored for 42 days had recoveries greater than 75% and complied with all the FDA criteria for RBC storage;⁵ as such, this solution has recently obtained

licensure approval for RBC transfusion therapy. Finally, we determined the long-term survival of RBCs that were still present in the circulation after 24 hours. The half-life survival of AS-7–stored RBCs was 33 ± 10 days for cells stored for 42 days and 31 ± 9 days for those stored for 56 days (Fig. 2F), which are within the published reference range of 28 to 35 days for conventional autologous infusions of RBC stored for shorter periods.³⁰

Because of the large number of donor units involved in this study, the data were examined for possibly mechanistic correlations between end-of-storage measures as previously published.³¹ At these high fractions of 24-hour recovery and survival, we observed no significant correlation between end of storage recovery with either hemolysis at 42 or 56 days or with RBC ATP concentration at 42 days of storage or even at 56 days.

DISCUSSION

AS-7 was designed to improve RBC storage by providing phosphate and bicarbonate to increase the storage system buffer range and capacity so as to support higher rates of RBC metabolism while keeping the final pH above values that are associated with programmed cell death. The solution appears to work as designed. In this study with 120 test and 60 control units, the system ameliorates many of the recognized cellular and metabolic features associated with the RBC storage lesion. RBCs stored in AS-7 for 42 days had higher 24-hour in vivo recovery than historic controls stored in the current US-licensed RBC ASs.^{5,8,32} Furthermore, longer storage of RBCs for up to 56 days in AS-7 maintains 24-hour recoveries over the minimal requirements of the US FDA. AS-7–stored RBC units had lower hemolysis and protein-rich RBC microvesicle shedding than concurrently stored AS-1 controls. Perhaps more important was the finding that all cells stored for up to 56 days had measured in vivo recoveries greater than 75% and hemolysis less than 1% , suggesting that the AS-7 prevented the number of poorly storing RBC typically seen in large RBC storage trials. All together these data indicate that long-term storage in AS-7 is robust and significantly slows the development of eryptosis in stored RBC.

More than 90 million units of RBCs are transfused worldwide every year. Transfusion of RBCs saves lives and enables the performance of life-saving medical and surgical therapies. RBCs are an essential medicine with an excellent therapeutic index.³³ However, studies have raised concerns about the safety of stored RBCs. Longer-stored RBCs have been implicated in increased morbidity and mortality in a number of retrospective clinical trials¹³⁻¹⁷ and meta-analyses.^{18,19} Recently, release of iron from stored and transfused RBCs has been shown to cause inflammation and increased mortality in animal models of transfusion.²⁰

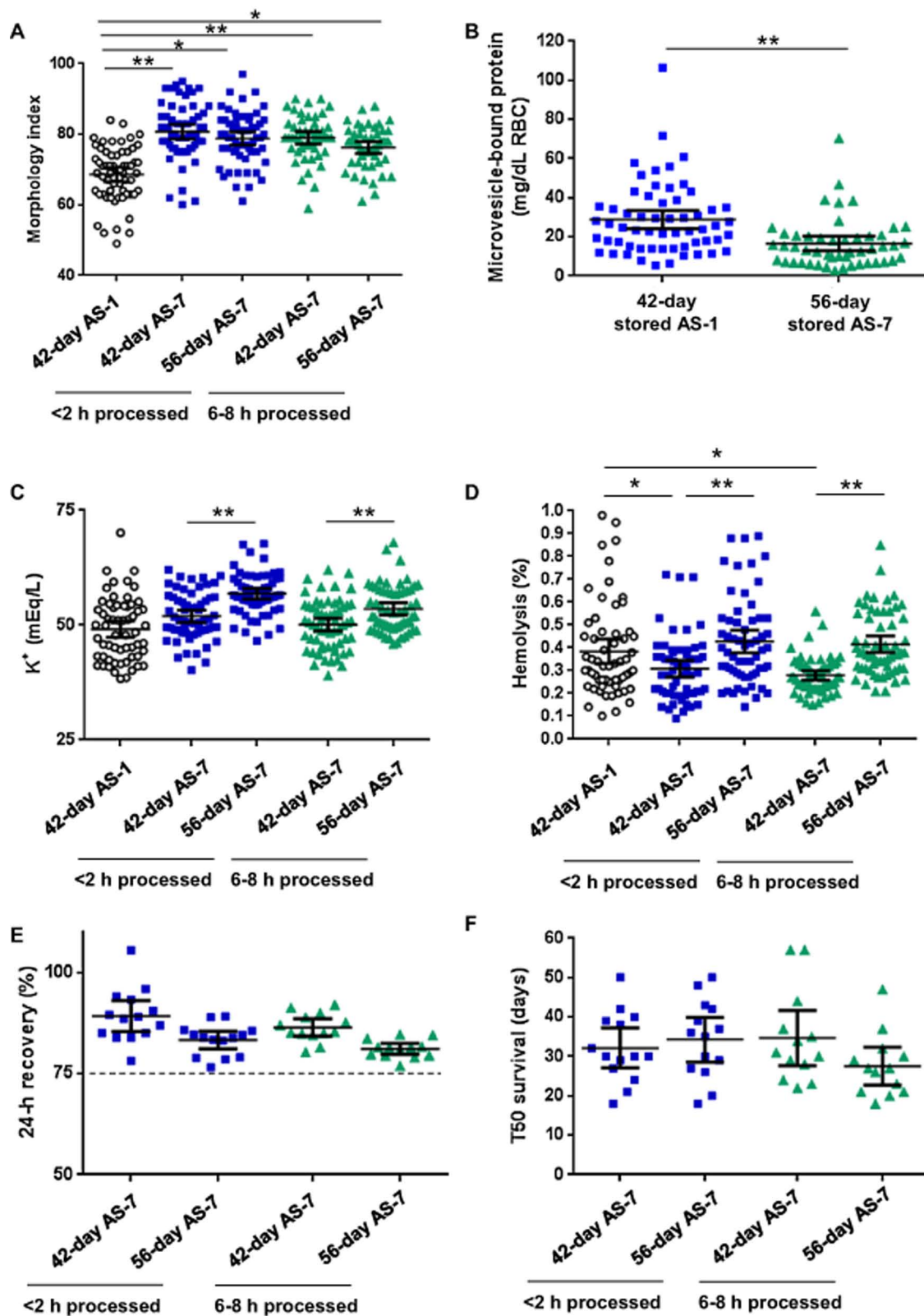


Fig. 2. Hemolysis, in vivo survival, and storage lesion predictors in 42- or 56-day-stored RBCs in AS-1 or AS-7. (A) Morphology index. (B) Microvesicle-bound protein in supernatant of RBC suspensions. (C) Extracellular potassium (K⁺) concentration. (D) Hemolysis. n = 60 per group. (E) 24-hour (h) recovery of ⁵¹Cr-radiolabeled RBCs. (F) Half-life (T50) survival of ⁵¹Cr-radiolabeled RBCs. n = 13 to 14 per group. Individual data are presented along mean $\pm$ 1 SD. *p < 0.05; **p < 0.01.

AS-7 represents a useful alternative to current RBC ASs while maintaining the volume of current blood products. The data presented in this report indicate that AS-7 better preserves the membrane integrity of RBC stored for up to 56 days than AS-1 does even at a shorter storage of 42 days. AS-7 increased the glycolytic flux through the stored RBCs, as measured by increased glucose consumption and increased lactate production with higher (approx. 45% average decreased concentration of hydrogen ions) end-of-storage pH and ATP concentrations. The increased glycolysis of AS-7-stored RBCs is probably a result of the activation of the key allosteric regulatory enzyme of glycolysis phosphofructokinase^{34,35} and relates to the increased buffer range of alkalized Hb residues and the greater buffer capacity associated with greater carbon dioxide loss.

AS-7 appears to achieve a greater buffer range mainly through higher initial intracellular pH associated with the chloride shift mechanism. Low chloride in the suspending AS and passive diffusion of chloride out of the cells results in a net loss of intercellular chloride. To maintain electrical neutrality, hydroxyl anions must diffuse into the cells in proportion to the chloride loss where they increase the intracellular pH and engage the high-capacity buffering of intracellular Hb.

With current concerns about the adverse effects of storage-injured RBCs in transfusion, AS-7 presents a clinically practicable way to reduce the RBC storage lesion and increase the quality of RBCs for transfusion, while maintaining or even expanding the availability of RBC products with only minor modifications in the current RBC production strategies or in the form and use of RBC products.

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CONFLICTS OF INTEREST

This study was sponsored by Hemerus Medical, LLC. Hemerus Medical, LLC, was acquired by Haemonetics, Inc., after the completion of this study. JAC, LJD, LAM, NR, LH, PW, AHS, and ZMS have disclosed no conflicts of interest. JRH has patent and royalty rights to AS-7 and is a consultant of Hemerus, LLC, and Haemonetics Corporation. MZ was an employee and

shareholder of Hemerus Medical, LLC, at the time of study and an employee of Haemonetics Corporation at the time of preparation of the manuscript.

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