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Metabolic effect of alkaline additives and guanosine/gluconate in storage solutions for packed red blood cells

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Abstract

Background—Over a century of advancements in the field of additive solutions for red blood cell storage has made transfusion therapy a safe and effective practice for millions of recipients worldwide. Still, storage in the blood bank results in the progressive accumulation of metabolic alterations, a phenomenon that is mitigated by storage in novel storage additives, such as alkaline additive solutions. While novel alkaline additive formulations have been proposed, no metabolomics characterization has been performed to date.

Study design and Methods—We performed UHPLC-MS metabolomics analyses of red blood cells stored in SAGM (standard additive in Europe), PAGGSM or alkaline additives SOLX, ESOL-5 and PAG3M for either 1, 21, 35 (end of shelf-life in The Netherlands) and 56 days.

Results—Alkaline additives (especially PAG3M) better preserved 2,3-diphosphoglycerate and adenosine triphosphate (ATP). Deaminated purines such as hypoxanthine were predictive of hemolysis and morphological alterations. Guanosine supplementation in PAGGSM and PAG3M fueled ATP generation by feeding into the non-oxidative pentose phosphate pathway via phosphoribolysis. Decreased urate to hypoxanthine ratios were observed in alkaline additives, suggestive of decreased generation of urate and hydrogen peroxide. Despite the many benefits observed in purine and redox metabolism, alkaline additives did not prevent accumulation of free fatty acids and oxidized byproducts, opening a window for future alkaline formulations including (lipophilic) antioxidants.

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Authors' Contributions ADA, RVB and DDK designed the study. HK, RVB, DDK generated the samples. RCH, JAR, ADA performed metabolomics analyses. ADA wrote the first draft of the manuscript and all the authors contributed to its finalization.

Disclosure of Conflict of interest ADA is a founder of Omix Technologies Inc. ADA is a consultant for New Health Sciences Inc. All the remaining authors have no conflicts of interest to disclose.

Conclusion—Alkalinization via different strategies (replacement of chloride anions with either high bicarbonate, high citrate/phosphate or membrane impermeant gluconate) results in different metabolic outcomes, which are superior to current canonical additives in all cases.

Keywords

mass spectrometry; metabolomics; transfusion medicine; chloride free; guanosine; gluconate

Introduction

One hundred years of advancements in the field of transfusion medicine and, in particular, storage additives for packed red blood cells (RBCs)¹⁻²⁻³ have made transfusion therapy a safe and effective mainstay of current medical practice for over 11 million Americans every year.⁴ In 1981 the first modern era additive containing saline, adenine glucose and mannitol (SAGM) was formulated. This allowed storage of packed RBCs for up to six weeks owing to the role of mannitol in balancing osmolarity and decreasing hemolysis.⁵ A similar additive solution (AS-1) to SAGM was subsequently formulated in the United States.⁶ AS-3 and AS-5⁶⁻⁸ were designed in the subsequent decades, with AS-3 containing phosphate buffers and citrate (instead of mannitol) to fuel synthesis of high energy phosphate compounds and make this additive more viable to pediatric patients, respectively. Over the years SAGM modifications have been formulated including guanosine as a source of ribose phosphate secondary to phosphoribolysis (PAGGSM), which thus sustains late ATP-generating glycolysis reactions without the need for a net expenditure of ATP.⁹ More recently, novel additives such as AS-7 (commercial name SOLX)¹⁰ or erythrosol-5 (ESOL5)¹¹ were designed building on the concept of chloride shift¹²⁻¹⁴ – which favors intracellular pH alkalinization by promoting chloride efflux in low chloride/chloride free/high bicarbonate additives - with beneficial effects to energy and redox metabolism (reviewed here).¹⁵ Similar effects could be obtained by replacing the saline component with salt containing membrane-impermeant anions such as sodium gluconate, which generates a reversible intracellular alkalosis.¹⁶⁻¹⁸

With over 112,5 million units donated globally every year and an average end of storage loss of potency of at least ~17%¹⁹ in current additives (calculated from post-transfusion recovery studies²⁰), novel storage additives still offer an actionable window of opportunity to significantly impact the field of transfusion medicine by improving RBC storage quality.²¹ Alterations of purine metabolism resulting from ATP consumption and breakdown into purines like hypoxanthine are markers of the metabolic age of packed RBCs.²² Decreases in the levels of non-deaminated purines cannot be corrected by additional adenine loading of storage additives.²³ Activation of purine deaminases such as erythrocyte-specific AMP deaminase 3 in turn results in potentially toxic byproducts such as hypoxanthine. Hypoxanthine has been recently identified as a negative predictor of post-transfusion recovery in stored mouse and human RBCs²⁴ and a substrate for circulating xanthine oxidase for the generation of urate and hydrogen peroxide.²⁵ In this view, end-of-storage rejuvenation of packed RBCs has been proposed, which exploits inosine and pyruvate to replenish late glycolysis via phosphoribolysis-derived pentose phosphate sugars and to promote oxidation of NADH back to NAD⁺ via lactate dehydrogenase, respectively.

However, excess inosine transformation to hypoxanthine results in the necessity to wash rejuvenated units prior to transfusion.²⁶

In addition, the use of alternative sugars (or sugar alcohols) instead of glucose, such as mannose, fructose or sorbitol (as a replacement for mannitol)^{27,28} has been proposed as a viable strategy to bypass early glycolytic blockade promoted by feedback inhibition of phosphofructokinase by intracellular acidification, a mechanism evolutionarily exploited by animals such as naked-mole rats to resist extreme anoxia.²⁹ Finally, antioxidants such as vitamin C, N-acetylcysteine and vitamin E have been proposed as additives for packed RBC storage in that they attenuate the oxidative storage lesion, especially to the lipid component, 30–34 which is relevant in the light of the correlation between lipid oxidation and post-transfusion recovery.³⁵

The current availability of new “omics” technologies is providing increased understanding of the RBC proteome and metabolome.^{36,37} For example, although RBCs lack mitochondria, they have now been shown to metabolize carboxylic acids from citrated anticoagulants or storage additives^{38,39} in an oxygen-dependent fashion.⁴⁰ Omics technologies have helped investigators in the field of transfusion medicine to characterize the multi-faceted nature of the storage lesion⁴¹ and, by qualitatively and quantitatively characterizing its evolution in different storage additives,^{42–45} pave the way for future developments in the field. Recently, we performed a comparative analysis of hemolysis, ion homeostasis and osmotic fragility of RBCs stored in five different additives.⁴⁶ Here we expand on those observations by providing metabolomics evidence of the beneficial effects of alkaline additives when compared to AS-3 and correlating metabolomics data to previously measured functional parameters.

Methods

Commercial reagents were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise noted.

Blood collection, processing and storage

Blood collection, processing and storage was described in detail elsewhere.⁴⁶ Briefly, 500 mL blood in CPD anticoagulant was collected from healthy, volunteer, donors (n=15) in accordance with the Declaration of Helsinki. For each series, three series of replicates consisting of 5 ABO-compatible pooled blood units were split, plasma and buffy coat-depleted. Subsequently 110 mL was added of the various additive solutions SAGM, E-SOL5, PAGGSM (Fresenius Kabi, Emmer-Compascuum, the Netherlands), SOL-X (AS-7) or PAGGGM (both prepared in house), followed by leukoreduction ($<1 \times 10^6$ remaining leukocytes). Formulations of the additive solutions are extensively described by Lagerberg et al.⁴⁶ Units were aseptically sampled at storage days 1, 21, 35 (end of shelf-life for RBCs in the Netherlands) and 56 days. The rationale behind this sampling timeline was informed by the observation that “the decline in 2,3 DPG was inhibited during storage in E-Sol 5 and AS-7, while in PAG3M, 2,3-DPG level increased above the initial level till day 35 and remained detectable till day 56”, as reported by Lagerber et al.⁴⁶ Here we wanted to test whether these observations for 2,3-DPG could be also expanded to other metabolic

pathways. RBCs and supernatants were separated by centrifugation at 2,000 *g* for 10 min at 4°C at each time point and the RBCs pellet were shipped frozen to the metabolomics facility to be processed for metabolomics analyses as described below.

Osmotic fragility, morphology, gas measurements were performed as previously reported.⁴⁶

Metabolite extraction

A volume of 50 µl of packed RBCs were extracted in 450 µl of lysis buffer (methanol:acetonitrile:water 5:3:2), before ice cold extraction by vortexing for 30 minutes at 4°C.^{38,47} Insoluble proteins were pelleted by centrifugation (10 minutes at 4°C and 10,000 x *g*) and supernatants were collected and stored at -80°C until analysis.

UHPLC-MS metabolomics

Analyses were performed using a Vanquish UHPLC system coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were resolved over a Kinetex C18 column (2.1 x 150 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) at 25°C using a three minute isocratic condition of 5% acetonitrile, 95% water, and 0.1% formic acid flowing at 250 µl/min,⁴⁸ or using a 9 min gradient at 400 µl/min from 5–95% B (A: water/0.1% formic acid; B: acetonitrile/0.1% formic acid).³⁸ MS analysis and data elaboration was performed as described.³⁸ Metabolite assignments were performed using MAVEN (Princeton, NJ, USA), as described.⁴⁸

Statistical analyses

Graphs and statistical analyses, including partial least square-discriminant analysis (PLS-DA) and Two factor (time series + one factor statistical analysis) were performed with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA) and Metaboanalyst 3.0.⁴⁹ Line plots were performed through interpolation of available data points for all tested storage days (third degree polynomials) via GraphPad Prism. Metabolic linkage analyses have been recently described.⁵⁰ Briefly, correlative analyses (Pearson or Spearman correlations – *r* – upon testing for normality distribution of data with Kolmogorov-Smirnov) and calculation of $|r| > 30\%$ deviations were performed with GraphPad Prism 5.0 and Microsoft Excel 2017, while results were plotted with GENE-E (Broad Institute, Boston, MA, USA). Briefly, the underlying assumption of the metabolic linkage analysis⁵⁰ is that – even though correlation does not necessarily imply causation - the levels of metabolites from linked pathways are highly correlated owing to biochemical constraints of the enzymatic reactions necessary to consume one metabolite to generate another.

Results

Gas and ion homeostasis, osmotic fragility, PS exposure and morphology

Hematocrit, mean cell volume (MCV), pH, % hemolysis, osmotic fragility, phosphatidylserine (PS) exposure and morphological alterations (reported as % of echinocytes) were measured for RBCs stored in different additives (in part previously reported⁴⁶ and here re-elaborated in Supplementary Figures 1 and 2). Though limited by the lack of functional *in vivo* measurements of RBC storage quality and transfusion efficacy

(e.g. post-transfusion recoveries), the parameters we measured here represent important surrogate indexes of “functional” relevance (e.g. % spontaneous and osmotic hemolysis). Mean cell volume (MCV) was highest in SAGM RBCs throughout storage (Supplementary Figure 2). SAGM RBCs had the highest end of storage osmotic fragility, with trends towards increase during storage duration (consistent with previous observations⁵¹), while other additives showed trends towards decrease (Supplementary Figure 1). Alkalinization in the various additives was achieved either by high bicarbonate load (SOLX), low chloride (PAGGSM) or no chloride (PA3GM, SOLX and ESOL-5 – Supplementary Figure 2). Lower end of storage pH was observed in PAG3M and PAGGSM in comparison to SOLX and ESOL-5, though trends were mostly comparable across all additives. ESOL-5 and PAG3M showed the lowest end of storage hemolysis and highest – though within a very narrow window range - PS exposure (the latter parameter normalized across additives by incubation at 37°C), despite all additives showing similar % of echinocytes upon morphological analysis throughout storage (Supplementary Figure 1).

Metabolomics – overview

Metabolomics analyses were performed at day 1, 21, 35 (end of standard storage in The Netherlands) and 56. Results are extensively reported in Supplementary Table 1. Hierarchical clustering of metabolic trends in all additives was performed (Supplementary Figure 3) and a detail of the top significant features (repeated measures ANOVA; $p < 0.01$ FDR corrected) is reported in Figure 1. On the basis of these metabolic phenotypes, we clustered samples via partial least square-discriminant analysis (PLS-DA – Figure 2.A; 2-dimensional plots for PC1 vs PC2, PC1 vs PC3 and PC2 vs PC3 are reported in Supplementary Figure 4), showing that PAG3M was the only additive not showing the typical U-shaped progression over PC1 and 2 previously described by us and others.^{22,43} Notably, glycolytic measurements through classic spectrophotometric approaches and UHPLC-MS (Figure 2.B and C, respectively) concordantly showed higher glucose consumption and generation of ATP, DPG and lactate in PAG3M, which contained half the initial dose of glucose as SOLX and ESOL5. In particular, despite of the lower intracellular pH, PAG3M better preserved DPG levels throughout storage, followed by SOLX and ESOL-5 (Figure 2.B). No significant alterations in reduced to oxidized glutathione ratios (GSH/GSSG) were observed across additives, though PAG3M showed the highest sedoheptulose phosphate/6-phosphogluconate ratio, while ESOL-5 the lowest (Figure 2.C). In the absence of flux data, this observation is suggestive of increased fluxes through the Pentose Phosphate Pathway (PPP) in PAG3M-stored RBCs, suggestive of higher NADPH-generating (i.e. oxidized thiol-reducing) capacity. ESOL-5 and SOLX had the highest levels of intracellular malate (declining in all additives over storage, likely owing to release in the supernatants), despite higher levels of citrate in the former additive (Figure 2.D).

Metabolic linkage analysis (Figure 3 - extensively described in previous reports⁵²) allows the determination of metabolic reprogramming of RBCs stored in different additives. The underlying rationale for this approach is that biochemical constraints of enzymatic reactions result in strong and significant positive/negative correlations across metabolites in the same pathways, unless the activity of one enzyme in the cascade is up/down-regulated by modulatory events (e.g. inactivating oxidation of active site cysteine of glyceraldehyde 3-

phosphate dehydrogenase is observed during RBC storage⁵³). Metabolite levels in SAGM RBCs were correlated to each other, prior to repeating a similar correlation analysis across all additives while maintaining the order of metabolites of original elaborations in SAGM (Figure 3.A) or performing a hierarchical clustering of correlations on an additive-by-additive fashion (Figure 3.B). This analysis provides at a glance an overview of metabolic rewiring across additives, indicating a strong effect on redox homeostasis, carboxylic acid, fatty acid and purine metabolism in alkaline additives when compared to SAGM (Figure 3). Of note, changes in glutamine metabolism and glutathione homeostasis were observed across additives with respect to total glutathione levels (both reduced and oxidized being lowest in PAGGSM – Supplementary Figure 5). SAGM showed the highest levels of GSSG for the first 21 days of storage. Cysteine accumulated significantly in ESOL-5 RBCs over storage duration in comparison to other additives, mainly at outdate of the unit at storage day 56 (Supplementary Figure 6).

Purine and fatty acid metabolism

Supplementation of guanosine in PAGGSM and PAG3M resulted in significantly higher levels of guanine and adenosine and comparably lower levels of inosine (especially PAG3M) in comparison to all the other additives tested here (Figure 4). This is suggestive of guanosine from PAGGSM and PAG3M being mostly metabolized via phosphoribolysis rather than hypoxanthine guanosine phosphoribosyl transferase (HGPRT). Deaminated purines (and the ratio of deaminated/non-deaminated purines – e.g. adenosine/inosine; AMP/IMP; adenine/hypoxanthine) were the lowest in PAG3M (Figure 4). On the other hand, urate to xanthine (or hypoxanthine) ratios were the highest in ESOL-5 and SAGM, followed by SOLX (Figure 4). Inferring from steady state data, these results are suggestive of increased purine deamination and catabolism to urate (a reaction that also generates the pro-oxidant hydrogen peroxide) in SAGM and ESOL-5, in part mitigated by SOLX.

On the other hand, SOLX showed the lowest accumulation of free fatty acids and arachidonic acid peroxidation products, followed by SAGM, PAGGSM, ESOL-5 and PAG3M (Supplementary Figure 3).

Correlation of metabolomics data to functional outcomes

Metabolomics data were correlated to functional outcomes (including energy state as determined by ATP levels, hemolysis, osmotic fragility, PS exposure and morphological alterations) in additive-dependent (Supplementary Table 1, Figures 5 and 6.B) and an additive independent fashion (Supplementary Figure 7, Figure 6.A). An overview of top metabolites showing significant positive (red) or negative (blue) correlations to ATP across all additives is provided in Figure 5.A. Of note, most trends observed showed a progression towards increasingly higher (e.g. lactate and ATP – Figure 5.B) or lower (e.g. ATP and sphingosine 1-phosphate) correlations across all additives (Figure 5.A). Correlations between ATP and DPG were poor for all additives except for PAG3M (Figure 5.C), suggesting a rewiring of the Rapoport-Luebering shunt specific to this additive. Of note, purine metabolites (especially ATP, adenosine and the deamination products inosine and hypoxanthine) were the best correlates with functional outcomes (Figure 6.B), with hypoxanthine being the best predictor of hemolysis and morphological alterations (%)

echinocytes) in all additives. Of note, inosine was a good predictor of osmotic fragility in all additives except for SAGM (Figure 6.B).

Discussion

In the last few years, metabolomics analyses of stored RBCs have generated a wealth of observational data whose clinical relevance is still matter of debate.⁵⁴ Based on the consideration that, in order to transport and deliver oxygen, transfused RBCs must remain intact and circulate in the bloodstream of the recipient, correlative studies have been published in mice and humans suggesting a role for lipid and purine oxidation (e.g. 5-HETE and other HETEs, ATP and hypoxanthine) in mediating RBC hemolysis and post-transfusion recovery.^{24,35,55,56} These observations are relevant in that they reconcile ex vivo metabolomics observations with potentially clinically-relevant outcomes. In this view, an improved additive solution for storage of packed RBCs should mitigate the metabolic lesion in order to reduce hemolysis and boost post-transfusion recoveries.

In the present study, we generated metabolomics data on 5 storage additives, including SAGM, the canonical European storage solution, and more recently introduced PAGGSM, as well as three alkaline additives (SOLX, ESOL-5 and PAG3M). Of note, results on SAGM and PAGGSM we report here are consistent with findings reported by Rolfsson and colleagues,³⁹ who also explored the metabolic impact of other non-alkaline additives such as AS-1 and AS-3, but did not focus on the impact of intracellular alkalinization on stored erythrocyte metabolism. Intracellular alkalinization is achieved in these additives by exploiting the chloride shift concept, by eliminating chloride anions from the formulation either balanced by high bicarbonate loading (SOLX), high citrate and phosphate (ESOL-5) or membrane-impermeant gluconate (PAG3M). Theoretically, the chloride-efflux effect induced by incorporation of gluconate in the additive should be reversible once anionic equilibrium is reached, unlike the chloride efflux induced by low/no chloride high bicarbonate additives. However, the chloride shift impact of gluconate (as determined by measurements of bicarbonate and chloride anions – Supplementary Figure 2) perfectly overlaps in the alkaline additives PAG3M, ESOL-5 and SOLX. Still, the gluconate containing additive (PAG3M) showed the highest DPG levels through the whole storage period, suggesting that phenomena other than alkalinization alone (i.e. fueling of late glycolysis by pentose phosphate compounds released via phosphoribolysis of guanosine) may contribute to the beneficial activation of the Rapoport-Luebering shunt. Classic biochemical understanding of RBC energy metabolism posits that the synthesis of 2,3-DPG through the Rapoport-Luebering shunt “sacrifices” the generation of one molecule of ATP.⁵⁷ While this held true also in the present study for all storage additives, including alkaline and guanosine containing additives, gluconate containing-PAG3M was the only additive showing significant positive correlations between the levels of ATP and 2,3-DPG, highlighting a metabolic peculiarity of RBCs stored in presence of the PAG3M formula.

Intracellular alkalinization is anticipated to boost glycolysis, the PPP and the Rapoport-Luebering pathway through the pH-dependent increases in the activity of the rate-limiting enzymes phosphofructokinase, glucose 6-phosphate dehydrogenase and bisphosphoglycerate mutase.⁴⁷ Here we show that decreased RBC spiculation (i.e. formation of morphologically

altered echinocytes) and hemolysis is observed in alkaline additives, a phenomenon that correlated with reduced ATP breakdown and purine deamination rather than free fatty acids and fatty acid oxidation. This observation, while preliminary, is suggestive of the fact that preserved energy homeostasis mitigates RBC spiculation, a “save or sacrifice” phenomenon to eliminate oxidation products (including oxidized lipids). However, preserved energy homeostasis in alkaline additives (especially PAG3M and ESOL-5) is not sufficient to completely prevent lipid oxidation, for which introduction of hydrophilic (e.g. ascorbate) or lipophilic (e.g. vitamin E) antioxidants may represent a better option.^{30–34} An alternative explanation to this phenomenon is an increase in oxidized lipid recycling through the Lands cycle, a phenomenon that is promoted by hypoxia⁵⁸ and may be similarly affected by alkalization.

On the other hand, alkaline additives effectively prevented purine deamination, especially in the case of PAG3M. Of note, introduction of guanosine to the additive formulation of PAGGSM and PAG3M ended up boosting the guanine nucleotide pool and fueling ATP generation with no initial energy investment in late glycolysis via phosphoribolysis, rather than generation of inosine through the activity of HGPRT. Lower levels of hypoxanthine and higher ratios of non-deaminated to deaminated purines (e.g. ATP/hypoxanthine) or decreased purine oxidation catabolites (urate/hypoxanthine) were indeed observed in all alkaline additives, especially in PAG3M, SOLX and ESOL-5 (in that order).

Increased phosphoribolysis may also explain the observed increases in steady state levels of non-oxidative phase PPP metabolites (e.g. sedoheptulose phosphate). This observation could also be alternatively explained by increased fluxes through the oxidative PPP, resulting in higher non-oxidative/oxidative PPP intermediate ratios (consistent with higher G6PD activity and thus increased antioxidant capacity in alkaline RBCs). Indeed, we recently appreciated the role that oxidative stress plays in activating AMP deaminase 3 to promote purine deamination and thus hypoxanthine accumulation, a phenomenon that negatively correlates with post-transfusion recovery and is prevented by hypoxic storage (which also induces intracellular alkalization).²⁴ However direct comparison of hypoxanthine levels in alkaline vs non-alkaline additives (SOLX vs AS-3) in unpaired studies showed increases in the former group, also accompanied by higher total levels of non-deaminated purines and ATP.⁴⁵ Similarly, supplementation of CO₂ to hypoxically-stored RBCs decreased hypoxanthine levels, though increased urate/hypoxanthine ratios. This suggests a role of alkalization in preventing hypoxanthine catabolism or a phenomenon independent from alkalization in explaining hypoxia-induced decreases in RBC hypoxanthine.⁵⁷ Similarly, it must be noted that – unlike RBCs – mitochondria-endowed cells generate hypoxanthine under hypoxic conditions, a caveat that would recommend caution when considering purine deamination-related data on hypoxic storage of RBCs in the presence of residual white blood cells (while the leukodepleted RBC units used for this study have $<0.1 \times 10^6$ leukocytes). Alternatively, ATP/hypoxanthine ratios, or hypoxanthine metabolism to urate and hydrogen peroxide, rather than hypoxanthine levels per se may be candidate markers of the RBC metabolic lesion for future studies. Finally, activation of purinergic signaling via adenosine receptors has been shown to boost RBC glycolysis and DPG generation in vivo and ex vivo, a phenomenon that may be leveraged to further promote purine homeostasis in stored RBCs under (alkaline) normoxic or hypoxic conditions.⁵⁹ Future additives may

contain antioxidants to promote energy metabolism via inhibition of purine deamination by redox sensitive deaminases, though antioxidants other than ascorbates or sugars other than glucose should be included in those formulations to avoid the negative effect on glycolysis triggered by competitive uptake of glucose and dehydroascorbate by GLUT-1 transporters.³⁰

Conclusion

In the present study we provided the first comparative metabolomics analysis of RBCs stored in different alkaline additives SOLX, ESOL-5 and PAG3M or in non-alkaline/classic additives such as SAGM or PAGGSM. The results from our metabolic analyses indicate superior RBC preservation in PAG3M as compared to the other alkaline additives and, in general, in alkaline additives as compared to non-alkaline SAGM and PAGGSM. In particular, DPG and ATP generation and maintenance, as well as purine metabolism and redox homeostasis (especially the PPP and its non-oxidative phase byproducts) were favorable in PAG3M and other alkaline additives in comparison to SAGM and PAGGSM. However, the observed metabolic benefits do not extend to the prevention of storage-induced fatty acid release and lipid oxidation. The correlation of metabolites from these pathways with surrogate ex vivo indexes of functional outcomes (e.g. cell morphology and osmotic fragility) are relevant in that they confirm and extend the recent observation on the (correlative) role of purine deamination in RBC recovery after transfusion.²⁴ However, additional functional in vivo measurements (e.g. post-transfusion recovery) will be instrumental to further expand our understanding of the impact of these storage additives on transfusion efficacy. Results from the present study provide additional insights in the metabolic benefits of alkaline storage additives and will likely guide the formulation of novel additives in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hierarchical clustering analyses of time course measurements of RBC metabolites at storage day 1, 21, 35 and 56 in five different storage additives
Significant metabolites by ANOVA (FDR corrected) are shown in the heat map (rows), upon Z-score normalization across all samples and color-coding (blue to red = low to high – legend in the top left corner). A vectorial version of this graph is provided as Supplementary Figure 3.

Figure 2. Partial least square-discriminant analysis (PLS-DA) of metabolomics data from RBCs stored in 5 additive solutions, following the color code on the right hand of panel A

In **B**, **C** and **D**, line plots indicate median $\pm$ range (continuous $\pm$ error bars) for metabolites involved in glycolysis as measured by classic spectrophotometric approaches (**B**) or UHPLC-MS (**C**), and carboxylic acids citrate and malate (**D**). Color codes are consistent with the legend in **A**.

Figure 3. Metabolic linkage analysis of RBCs stored in five different additive solutions

In A, metabolites were correlated to each other and results plotted as correlation maps (from blue to red = r from -1 to $+1$; Spearman's correlation). Metabolites were thus hierarchically clustered on the basis of Spearman correlations of all values at any given time point as measured in SAGM. The order of metabolites was thus maintained across all additives, though Spearman's correlations were calculated separately for each additive in order to visually show disruption of metabolic linkage⁵² (correlation across metabolites) across the different storage conditions. This analysis allows identification of the disruption of correlations and generation of new ones in response to storage in different additives, an indirect measure of metabolic rewiring. A hypothetical example of two metabolites (x and y) correlating differently between each other under a condition AS-X – in red – and AS-Y (in blue) is provided in the bottom left corner of the figure. In B, hierarchical clustering of Spearman correlations across all metabolites was independently performed for each additive solution to reveal the formation of new clusters of highly correlated metabolites in alkaline additives involving metabolites from redox homeostasis, carboxylic acids, fatty acid and purine metabolism.

Figure 4. Purine metabolism

In A, line plots indicate median $\pm$ range (continuous $\pm$ error bars) for metabolites involved in purine metabolism as measured by UHPLC-MS. Color codes are indicated in the bottom right corner of panel A. In B, an overview of the pathway involving the metabolites graphed in A.

Figure 5. Top metabolic correlates to ATP across all additive solutions, plotted as a hierarchically clustered heat map (A)

In **B** and **C**, linear Spearman correlations (numbers in each panel) of lactate and 2,3-DPG to ATP in each one of the additive, graphed according to the color code in **C**.

Figure 6. Correlation matrix of all metabolites to outcomes (A) and highlight of top metabolic correlates to hemolysis, morphology and osmotic fragility across all additives
Purines, especially adenosine and its deamination products inosine and hypoxanthine were the best correlates to all tested functional outcomes independently (top row) or dependently (bottom row) on the additive solution. Color code in the bottom right corner of the figure identifies linear correlation curves across all additives at all tested time points.