Time to revisit red blood cell additive solutions and storage conditions: a role for "omics" analyses

Rosemary L. Sparrow

Research and Development, Australian Red Cross Blood Service, Melbourne, Victoria, Australia

Red blood cell (RBC) transfusion is a critical, life-saving treatment for severe anaemia caused by disease or chemotherapy, or by blood loss due to trauma or major surgery. For several decades RBC components have been prepared as concentrates suspended in nutrient additive solution, which preserves and extends the shelf-life of the RBC component, allowing up to 6-7 weeks of refrigerated storage[1]. Nevertheless, during storage RBCs undergo a complex and progressive accumulation of physicochemical changes, collectively referred to as the RBC storage lesion[2,3]. Recent clinical studies have identified RBC transfusion as an independent risk factor for increased morbidities and mortalities in certain groups of patients, including trauma, cardiac surgery and the critically-ill (reviewed in[4-6]). Additionally, some of these studies have identified that older stored RBCs are more strongly implicated in poorer outcomes compared to fresher RBCs[6]. In order to address these concerns, there is renewed interest to better understand the RBC storage lesion and to find ways to ameliorate the deleterious effects of storage, thereby improving the quality, efficacy and safety of RBC components for all transfusion recipients.

While increased research effort is being directed to better understand the effects of storage on RBCs and the potential impact on transfusion outcomes[7], slower progress is being made in finding ways to deter the detrimental effects of the RBC storage lesion. This perspectives paper will focus on this latter aspect and will provide a brief overview of the currently licensed RBC additive solutions, new experimental additive solutions, some of the challenges for progressing the development of RBC storage systems and where the application of "omics" analyses could benefit the advancement of RBC storage systems.

RBC additive solutions - old and new

The first RBC additive solution, saline-adenine-glucose (SAG) was developed by European researchers in the late 1970's[8]. Soon after, in 1981, the same researchers added mannitol to help protect the RBC membrane and reduce hemolysis, which enabled up to 6 weeks refrigerated storage of RBCs at a haematocrit of approximately 55 to 60 percent[9]. This modified SAG formulation was named SAGM and to this day SAGM is the most widely used RBC additive solution (Table I). Several countries apply a shorter 5 weeks shelf-life to their SAGM-RBC components despite the fact that SAGM is approved for 6 weeks storage. SAGM has not been licensed by the Food and Drug Administration (FDA), and hence is not used in the USA. Other additive solutions, which are all essentially variations of SAG/SAGM, have since been developed and commercialised, including AS-1, AS-3, AS-5, MAP and PAGGSM[10-13] (Table I). These solutions tend to provide improved preservation of RBCs compared to RBCs stored in SAGM, such as decreased haemolysis and reduced shedding of microparticles from the RBCs[14-16]. Nevertheless, the specific biological mechanisms that are modulated in RBCs stored in the variant SAG/SAGM solutions have not been precisely identified. A more detailed history of the development of RBC additive solutions can be found elsewhere[1]. In summary, no new RBC additive solutions have been licensed for use for over 20 years.

All of the currently licensed RBC additive solutions have an acidic pH (~5.6-5.8), which is well below the normal physiological pH of 7.3 of venous blood. Acidic additive solutions (and anti-coagulants) are used simply because it is easier to heat-sterilise a glucose-containing solution at an acidic pH. At physiological and alkaline pH, glucose caramelises during heat-sterilization. RBCs have sufficient buffering capacity to adjust the pH to closer to physiological levels during the first few days of storage in an acidic environment. However the buffering capacity of the RBCs is soon exhausted due to the generation of lactic acid by the RBCs via the anaerobic glycolytic pathway. Consequently

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Table I - RBC additive solutions currently in routine use around the world.

Constituents (mM)	Licensed RBC additive solutions					
	SAGM	**AS-1 Adsol Baxter**	**AS-3 Nutricel Pall Medical**	**AS-5 Optisol Terumo**	**MAP**	**PAGGSM MacoPharma**
NaCl	150	154	70	150	85	72
$NaHCO_3$	-	-	-	-	-	-
Na_2HPO_4	-	-	-	-	-	16
NaH_2PO_4	-	-	23	-	6	8
Citric acid			2	-	1	
Na-citrate	-	-	23	-	5	-
Adenine	1.25	2	2	2.2	1.5	1.4
Guanosine	-	-	-	-	-	1.4
Dextrose (glucose)	45	111	55	45	40	47
Mannitol	30	41	-	45.5	80	55
pH	5.7	5.5	5.8	5.5	5.7	5.7
Anti-coagulant	CPD	CPD	CP2D	CPD	ACD	CPD
FDA Licensed	No	Yes	Yes	Yes	No	No
Countries used	Europe UK Australia Canada New Zealand	USA	USA Canada	USA	Japan	Germany

the extracellular and intracellular pH of RBCs progressively becomes more acidic during storage, reaching a pH of ~6.5 after 6 weeks of storage in acidic additive solutions[17].

An acidic intracellular environment alters the activity of certain enzymes and biochemical pathways. For RBCs, increased intracellular acidity interferes with the generation of adenosine 5′-triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG), which are crucial for the RBC's survival and function in delivering oxygen, respectively. During storage of RBCs in acidic additive solutions, the intracellular concentration of ATP and 2,3-DPG decline[17]. Over the past 15 - 20 years, research into the development of new additive solutions has focussed on ways to maintain higher intracellular levels of ATP and 2,3-DPG during storage of RBC components[1]. A significant driver for this research came from the military, who were keen to find ways to store RBCs for longer than 6 weeks, thereby easing blood component inventory management and supply to battlefield hospitals and remote areas cut-off from regular shipments of fresh blood supplies. Longer storage time is not a key interest to the civilian blood services, and with the current concerns about the "age of blood"[6,7], there is no impetus to extend the shelf-life of RBC components. Nevertheless, there should be a desire by the civilian blood services and the medical community to support new technologies, including new RBC additive solutions that offer further improvement to the quality and efficacy of RBC components that will benefit their customers, the transfusion recipients.

Of particular promise are the alkaline, chloride-free, hypotonic solutions, which have been the subject of research by several groups over the past 10-20 years[1,18,19]. The chloride-free formulations rely on a "chloride shift" to establish a Donnan equilibrium between the charged ions in the intracellular and extracellular medium[2,18]. Thus in a chloride-free medium, intracellular chloride will leave the cell and in the absence of any other diffusible anion, hydroxide will enter the cell and raise the intracellular pH. The alkaline storage solutions contain glucose and

thus have to be sterilised as a "two-pack" system to prevent glucose caramelisation; one pack contains the alkaline salts and the other pack contains the glucose solution, which are combined at the time of RBC component manufacture. After extensive testing of numerous variants, experimental formulations have been devised that appear to maintain RBC quality better than the conventional additive solutions, giving at least 2 weeks advantage to RBCs stored in the new generation additive solutions[19,20].

RBC storage research - expect the unexpected

Despite the RBC having been a favourite experimental model for cellular biologists and biochemists, RBC storage research has repeatedly demonstrated that a lot of fundamental biology about RBCs is still not well understood. The complexity of the inter-relationship between RBC biochemistry, cytoskeletal structure and membrane properties have made it difficult to predict how RBCs will respond to different storage conditions. Exposure of RBCs to non-physiological storage environments has pointed to the existence of previously unknown biochemical mechanisms in RBCs, including apoptotic-like processes, ion and osmotic channels that behave differently than expected, exposure of new or altered receptors possibly due to oxidative and/or protease/glycosidase activities or altered senescence[21-24].

"Expect the unexpected" is a fitting motto for researchers working in the field of RBC storage. An example is a recent study that showed that RBCs stored in an alkaline, chloride-free additive solution had higher intracellular concentrations of ATP and 2,3-DPG, as expected, but unexpectedly this did not appear to be related to the intracellular pH, which became acidic within the first week of storage[25]. Therefore the premise that the "chloride shift" operates by increasing the intracellular pH of RBCs may need to be refined[18]. Similarly, we have observed unexpected responses by RBCs to storage in an experimental hypotonic alkaline, chloride-free additive solution in that RBCs appeared to dehydrate[15]. This suggests that the ion flux into and out of the RBCs stored under "non-physiological" storage conditions caused altered behaviour of the ion channels and co-transporters that were not readily predictable based on current knowledge. Much is still to be learned about the effects of storage conditions on RBCs.

Challenges for the introduction of new RBC storage systems

The benefits gained by improved RBC component quality should more than justify any real or perceived inconvenience to the blood services in implementing adjustments to their processing procedures or additional processing costs of the introduction of new generation RBC additive solutions. The bigger challenge that has hindered the advancement of this field is the significant financial burden and risk for manufacturers of blood collection systems to obtain licensure and to bring a new RBC storage system to a market that is inherently based on very low profit margins, such as the blood services sector.

The financial burden to technology developers of new RBC storage systems is largely due to regulatory requirements, particularly those mandated by the FDA. In addition to *in vitro* data, the FDA requires *in vivo* data on the 24 hours post transfusion recovery of transfused autologous RBCs. Recently the FDA has tightened and increased the assessment and acceptance criteria making it potentially more difficult and expensive to bring new RBC storage systems to market. Although the regulatory agencies are to be commended for focussing on the safety of new therapies and devices for patients, there are concerns that the regulatory requirements for RBC storage systems have become excessive and are hindering progress (see an excellent discussion paper by Hess[26]).

Another significant challenge for obtaining licensure of new RBC storage systems is the inherent donor-related variability in stored RBC quality. It has long been recognised that RBCs from some donors do not store well, as evidenced by higher levels of haemolysis at RBC component expiry[14] and poorer *in vivo* 24 hr recovery data[27]. The relationship of specific donors and poorer quality of some stored RBC components was confirmed in a recent paired cross-over study designed to compare manual and automated whole blood processing methods[28]. Technology developers are unwilling to take on the risk that a random poor quality RBC component could jeopardise the success of licensure tests and clinical trials of their new blood storage systems and their significant financial investment.

The donor-specific factors that contribute to the storage quality of RBC components are yet to

be identified. Genetic, undiagnosed/sub-clinical medical conditions and lifestyle factors are all likely to be involved, although not evident by the donor's health or haematological status at blood donation. RBCs from some donors may be more susceptible to oxidative and/or mechanical stresses encountered during *in vitro* processing and storage. Some evidence suggests that RBCs from young female donors are more resilient to mechanical stress than RBCs from male donors and post-menopausal women[29]. This may relate to hormonal influences of the female menstrual cycle[30]. Lifestyle including diet, exercise, alcohol consumption and smoking may also contribute to RBC susceptibility to oxidative and/or mechanical stresses[31-33].

What can "omics" analyses do to advance the field?

Two areas of scientific endeavour have been identified here where the input of "omics" expertise could benefit the development of improved RBC components for transfusion: 1) to better understand the influence of RBC additive solutions and storage conditions on RBC metabolic pathways, function, quality and survival; 2) to identify the donor-specific factors that influence the quality of stored RBCs and how these factors affect RBCs.

Several research groups have made in-roads into cataloguing the effect of storage on the RBC proteome by relatively "untargeted" approaches[21,24,34]. Proteomics investigations that encompass identification of altered proteins indicative of cleavage, oxidative damage and/or glycolytic modifications as well as relative abundance can provide invaluable information about the effect of storage conditions on RBCs[21,35]. Some preliminary metabolomics analyses of stored SAGM-RBCs have been reported recently, indicating that such approaches are feasible to map the biochemical changes that occur to RBCs during refrigerated storage[36,37]. Technologies and data processing software to undertake sophisticated and detailed metabolomics analyses are now available and these could be used to fully map the biochemical changes to RBC proteins, lipids and carbohydrates during storage of RBC components to compare the effects of different additive solutions and storage conditions. Likewise, "omics" analyses could assist in identifying the biological mechanisms by which donor-specific factors affect the quality of stored RBC components. All of this information could guide the refinement of RBC storage systems, maximise inventory management and further improve the quality, safety and efficacy of RBC components for transfusion. It is now time to harness the power of "omics" technologies to find answers and remedies for some of the specific scientific issues and concerns about stored RBC transfusion components.

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Correspondence: Rosemary L Sparrow
Department of Immunology, Monash University
Research and Development, Australian Red Cross Blood Service
PO Box 354
South Melbourne VIC 3205, Australia
e-mail: rsparrow@ redcrossblood.org.au