Established and theoretical factors to consider in assessing the red cell storage lesion

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The collection and storage of red blood cells (RBCs) is a logistical necessity to provide sufficient blood products. However, RBC storage is an unnatural state, resulting in complicated biological changes, referred to collectively as the "storage lesion." Specifics of the storage lesion have been studied for decades, including alterations to cellular properties, morphology, molecular biology of carbohydrates, proteins and lipids, and basic metabolism. Recently, mass spectrometry-based "omics" technology has been applied to the RBC storage lesion, resulting in many new observations, the initial effects of which are more information than understanding. Meanwhile, clinical research on RBC transfusion is considering both the efficacy and also the potential untoward effects of transfusing stored RBCs of different ages and storage conditions. The myriad biological changes that have now been observed during the storage lesion have been extensively reviewed elsewhere. This article focuses rather on an analysis of our current understanding of the biological effects of different elements of the storage lesion, in the context of evolving new clinical understanding. A synopsis is presented of both established and theoretical considerations of the RBC storage lesion and ongoing efforts to create a safer and more efficacious product. (Blood. 2015;125(14):2185-2190)

Biology of RBC storage: effect upon the red cell and effects upon the patient

The practice of storing red blood cells (RBCs) prior to transfusion has been in place for close to a century.1,2 Indeed, the storage and banking of RBCs is a necessity to provide a sufficient volume of RBCs for patient needs. However, banking blood is an unnatural state for RBCs to exist; sitting in the static environment of a refrigerator shelf, in a solution of glucose, citrate, and other additives, in the absence of a kidney or liver to detoxify products of ongoing metabolism and likewise with no reticuloendothelial system to remove senescent or damaged RBCs. Moreover, additional factors are introduced, such as plasticizers from the bag, which both inadvertently not only improve RBC integrity3,4 but also introduce concerns regarding toxicity.5,6 In this artificial environment, it is thus not surprising that stored RBCs undergo myriad changes, which are in aggregate referred to as the "storage lesion."

In recent years, the scope of clinical concern regarding transfusion of stored RBCs has widened from traditional issues of replacing lost RBCs with stored RBCs that could deliver oxygen to peripheral tissues, to concerns regarding the accumulation of toxicological entities in stored RBCs that could lead to medical sequelae upon transfusion. Much like the difficulties in studying tissue oxygenation by RBCs, analyzing sequelae of transfusing stored RBCs (if such sequelae even exist) is a very challenging process.7 Retrospective studies have been useful, but give a wide variety of results, with a large number of studies showing worse medical outcomes as a function of RBC storage time, others showing no effect, and some reporting worse outcomes with fresh RBCs. Although of great potential utility from the standpoint of using existing medical data to generate observational hypotheses, retrospective studies inevitably suffer a series of intrinsic biases. For this reason, several prospective randomized clinical trials (RCTs) were launched,3,8,9 of which have now reported no difference between groups receiving fresher vs older blood. The Age of Red Blood Cells in Premature Infants (ARIPI) study detected no difference in fresher vs older RBC units in very low-birth-weight infants,9 whereas the Red Cell Storage Duration Study (RECESS)10 recently reported no difference in cardiac surgery patients. Likewise, the Age of Blood Evaluation (ABLE) trial recently reported no difference in intensive care unit patients.11 Formal publication of the results of RECESS and ABLE (outside of abstract form) have not yet occurred and additional trials remain under way.8,12

RCTs are of tremendous use in helping to resolve this important issue. Although it is inevitable that some challenges to methodologies and analysis will be raised, these trials nevertheless provide very reassuring data indicating that large effects are not present in the particular patient populations that have been studied, with the RBC storage methodologies being used, and with the definitions of old vs fresh blood that were used. However, an additional concern remains, due to the tremendous number of transfused patients. In the United States alone, ~5 000 000 patients receive an RBC transfusion each year; thus, a difference as little as 1% would equate to 50 000 patients annually. Regrettably, the cost and logistical difficulties of carrying out RCTs is such that trials powered to detect such small outcomes are unlikely to be forthcoming.13 Conversely, this means that large and clinically relevant improvements would need to be made to RBC storage conditions before any new RBC products could be demonstrated to be superior to current products based upon RCTs.

Potential toxicity of transfusing stored RBCs

There are substantial data to indicate that among the substances that accumulate during RBC storage, a number of entities are generated with...
both theoretical and actual toxicology in the context of in vitro human and whole animal models. Such substances include:

- scavengers of nitric oxide (NO) and inhibitors of NO generation that may result in lack of vascular relaxation (eg, hemoglobin, both free and in microparticles, alterations in RBCs themselves, asymmetric dimethylarginine that may inhibit NO synthase);
- microparticles and RBCs themselves that may have procoagulant effects and/or other effects on innate and/or adaptive immunity and also physiology;
- free and nontransferrin bound iron that may promote growth of siderophile bacteria;
- clearance of damaged RBCs that may result in activation of innate immunity;
- the generation of bioactive lipids that prime neutrophils and may contribute to transfusion-related acute lung injury, and alteration in RBCs.

In some cases, the biological effects of stored/aged blood in whole animal models has resulted in profound pathology and pathophysiology, with substantial morbidity and mortality of recipients of the stored (but not fresh) units of blood. Such animal studies must be interpreted with a number of caveats, as animal biology may not reflect human biology and such studies are generated to maximize potential effects (ie, recipients of stored RBCs get all units at outdate and receive large volumes of blood). Like any system, the toxicology of animal studies has titratable dose response curves, and toxicological effects are confined to dose ranges that likewise may not translate to most humans.

The ARIP1, RECESS, and ABLE trials described above bring substantial intellectual comfort that within the context of our current practice and standard of care, there are not large effects between groups receiving “fresher” vs “older” stored RBCs. Nevertheless, these RCTs must likewise be interpreted with their own caveats, including:

1. the limited power to detect small effects (even below 20% depending upon the nature of the storage lesion),
2. the fact that few patients in these trials received multiple units of blood at outdate but, at some frequency, that such patients likely exist in real practice, and
3. that just as animal biology may not reflect human biology, the clinical susceptibilities of the patients in the existing RCTs may not reflect susceptibilities in other patient populations.

Thus, both the animal studies and the human RCTs are compelling and essential parts of an evolving landscape of data, and must be given the intellectual influence and weight they are due, without overinferring or generalizing to unobserved situations to which they may not apply.

Traditional measures of RBC storage: strengths and weaknesses

Primary metrics that have historically guided development and refinement of RBC storage conditions have focused on RBC cellular integrity. RBCs that hemolyze in the storage bag are clearly no longer viable as a therapy for anemia; thus, the US Food and Drug Administration (FDA) requires that on average, hemolysis in the bag be <1%. However, just because an RBC is intact does not mean it will circulate upon transfusion. Accordingly, the number of RBCs that remain in circulation 24 hours posttransfusion has been a primary criteria for the conditions and length of RBC storage that the FDA will approve (there must be a mean “24-hour recovery” of at least 75% with a standard deviation of <9% and with a 1-sided 95% lower confidence limit for the population proportion of successes of >70%). The cutoff of 75% is based upon historical expert opinion, but is nevertheless an arbitrary metric. The rational basis for the 24-hour recovery is that the removal of irrevocably damaged RBCs appears to occur within this 24-hour window, and RBCs which are still circulating 24 hours posttransfusion have a normal RBC circulatory lifespan.

It seems a fair statement that RBCs which do not circulate cannot deliver oxygen; thus, using 24-hour recoveries is a meaningful metric. However, even the word “circulate” requires certain biological scrutiny in this context. It might seem a reasonable conclusion that if a labeled RBC is injected IV and 24 hours later is recovered from a peripheral blood draw, it has been circulating. It might also seem a reasonable conclusion that if 75% of transfused RBCs are recovered 24 hours after infusion, then 25% of the transfused RBCs are no longer circulating. However, both of these assumptions have undergone a critical evaluation. With regards to the first notion, it has been rightly pointed out that the flow dynamics of large vessels are profoundly different than issues of the microvasculature of capillary beds. As the vast majority of oxygen exchange occurs in the microvasculature embedded in organ parenchyma, it is the actual behavior of RBCs in microcirculation that likely affect oxygen delivery capacity. It is for this reason that a great deal of study has gone into the rheological properties of RBCs, using a variety of instruments that measure RBC flexibility, the ability to bend and contort, and the ability to navigate artificial microvasculature with a variety of properties meant to model capillaries. Indeed, stored RBCs show progressively diminished flexibility and ability to perform the contortions necessary for microcirculation and show negative effects upon circulation itself; although studies to the contrary have also been reported. Moreover, transfusion of RBCs stored for longer periods has a statistically significant inverse correlation with both 24-hour posttransfusion, presumably due to some manner of adhesion or sequestration.

Regrettably, just because an RBC can circulate (even through the microvasculature), does not mean that it is in fact capable of delivering oxygen with normal efficiency. Indeed, there are data to indicate that stored RBCs may not optimally deliver oxygen until they have “recovered” after a period of time circulating in the recipient. This issue remains a matter of some dispute, with evidence on both sides; however, what the experimental data do demonstrate is that RBCs are capable of a phenotype in which they circulate but have decreased efficiency of oxygen delivery. Thus, this must remain a concern in guiding development of RBC storage conditions and has led to the view by some that stored RBCs do not function well. An argument that is often forwarded to the contrary is that many trauma patients who have had their entire blood volume replaced with stored RBCs survive, and patients requiring exchange transfusion do better clinically, indicating that stored RBCs can function to a reasonable extent. This seems undeniably true, and is a credit to the efficacy of transfusing stored RBCs. However, the fact that patients are routinely transfused with stored RBCs and many have good outcomes cannot logically justify the claim that stored RBCs function optimally nor does it demonstrate that stored RBCs are not damaging to patients in some ways.

Early characterization of the storage lesion focused on a number of parameters for which there was a rational basis to assume that they would reflect RBC function, including: (1) metabolic changes (eg, adenosine triphosphate [ATP], 2,3-diphosphoglycerate, etc), (2) enzymatic changes, (3) changes in rheological properties, and (4) physiologic
changes (eg, shape change, membrane remodeling), which have been recently reviewed.\textsuperscript{51,52} Oxidative changes have also been thoroughly characterized, have been shown to correlate in many ways with listed items 1 through 4, and have been posited as playing a causal role in the RBC storage lesion.\textsuperscript{51,52} However, although it seems a reasonable prediction that some of the above metrics would predict how stored RBCs would perform after transfusion, such is regrettably not the case.

A major challenge to the field has been that although the above measures do reflect observable alterations in RBCs during storage, they have only an “asymmetric correlation” to the ability of RBCs to survive storage (as measured by 24-hour recoveries). In other words, RBCs with extreme changes can be shown to have poor 24-hour recoveries; however, none or a small amount of change does not necessarily predict good 24-hour recoveries. Such is even the case for ATP levels, which have been a mainstay measure of RBC storage quality and have guided the addition of adenine to RBC storage solutions. Indeed, ATP at the end of storage has been the best predictor of 24-hour RBC recoveries; however, even ATP has only a 40% correlation with 24-hour RBC recoveries (with a mean 75% to 80% recovery).\textsuperscript{55} This poor correlation may be in part due to intrinsic difficulties in measurements of high accuracy,\textsuperscript{54} but may likewise reflect a lack of biological correlation. The lack of correlation between in vitro measures and in vivo circulation raises the serious concern that current metrics may be unthethered to relevant biology.

**Biological properties of RBCs other than carrying oxygen: more recent metrics of RBC storage**

In more recent decades, the concept of in vivo RBC function has evolved well beyond the issue of oxygen delivery. The current paradigms now include a role of RBCs as not only a vehicle of oxygen delivery, but also as both an agent of CO\textsubscript{2} removal, a sensor of tissue oxygenation, and a regulator of biological processes extrinsic to the RBC (eg, vascular tone through NO biology).\textsuperscript{55} Thus, additional metrics of RBC storage have been added to the above-mentioned details, including distinguishing hemoglobin outside of intact RBCs (eg, either free or in the form of microparticles that may scavenge NO), the amount of S-nitrosylation of hemoglobin itself (SNO-Hg),\textsuperscript{59} adhesion of RBCs to endothelial beds,\textsuperscript{56} effects of stored RBCs on models of vascular tone (eg, isolated aortic rings),\textsuperscript{57} and in vivo measures of how stored RBCs affect vascular tone, both in animals and directly in humans.\textsuperscript{59} However, as with the long list of more traditional measures, the clinical meaning of these indications remains largely undetermined (and in some cases controversial) and the ability to predict RBC function is unclear.

**Arrival of the “omics generation”**

The era of “omics” biology has now made its way to the study of the RBC storage lesion.\textsuperscript{59} As in many fields, the application of “big data” platforms (eg, proteomics, lipomics, glycomics, and metabolomics) has led to a progression toward a seemingly encyclopedic list of the changes that take place to RBCs over 42 days of storage.\textsuperscript{53,60-71} Genomics of RBC storage is a bit more abstract, as mature RBCs do not have nuclei and do not express gene products; however, it is well known that there is substantial variation in RBC storage across donors and it has also been reported that some characteristics of RBC storage are heritable traits in humans.\textsuperscript{68,69} The generation of such large quantities of data are of great potential, and have led to the ability to rapidly characterize storage biology to greater depths. These studies have detected an entire panoply of new molecular changes that correlate with RBC age, as well as confirming historical observations. Importantly, such data have also allowed systems biology approaches to RBC storage biology, which will likely be of great scientific utility moving forward.\textsuperscript{72} However, like the older measures, it is as yet unclear which, if any, of the new measurements correlate with the in vivo behavior of the RBCs. Thus, for the time being (at least), the generation of large volumes of new data has only exacerbated the central problem of which variables to study. Thus, a downside to the new data are that given the thousands of changes RBCs undergo during storage, and the experimental difficulty (or impossibility) of changing and testing a given variable in isolation from other variables, the actual significance of any of the observed changes remains unassessed by experimental methodologies and thus remains somewhat obscure.

So, the question is raised, how will the field move forward in sorting out the proverbial wheat from the chaff? One pathway is through experimental modification of storage solutions that give a better RBC product, as assessed by traditional metrics, if not better medical outcomes. Analysis of what variables correlate with a better RBC storage solution, and which do not, will not isolate individual changes, but will nevertheless help to establish the functional significance of at least different groups of changes. Controlled animal experimentation will also be of use in helping to isolate pathways, and will certainly provide a much greater degree of experimental control than can be ethically or practically achieved in humans, with the decidedly negative bedfellow that animal RBC biology will differ from human RBC biology to an as yet unknown extent.\textsuperscript{59} Thus, animal studies will likely find their greatest utility in sorting through the multiplicity of known changes in discovery-phase searches that will then allow more focused hypothesis testing in humans. Of central importance will be the combination of omics analysis with units of RBCs that are subsequently transfused into humans, and at the very least subjected to traditional measures of in the bag hemolysis and 24-hour recoveries. First steps to this end have been taken in platelet storage biology, and have isolated initial candidates for changes that predict posttransfusion performance.\textsuperscript{73-76} Similar approaches to RBC storage are likely to bear similar fruits, and seem a logical next step.

**Theoretical considerations on predicting likely variables and pathways of importance**

It is clear that certain storage lesion–based changes correlate with poor RBC performance (eg, hemolysis, extreme changes in morphology [echinospherocytosis], high levels of protein and lipid oxidation, loss of sialic acid, cross-linking of hemoglobin to itself and other proteins, clustering of band 3, etc). The significance of such changes may be somewhat exaggerated in the context of confusing the distinction between that which can occur and that which clearly does occur. Just as one example, the observation that transfused RBCs clear rapidly if first treated with neuraminidase (which removes terminal sialic acids from glycans) does indicate that if sialic acids are lost then RBCs will clear; however, this does not equate to establishing that the degree of spontaneous sialic acid loss during storage is sufficient to result in RBC clearance. This is an issue that affects essentially all of the proposed mechanisms of poststorage clearance of damaged/aged RBCs. Unless and until a variable can be reversed in isolation from other variables, and the effects of such reversal can be assessed in vivo, then the requirement of a given pathway cannot be firmly tested. Such maneuvers are technically challenging, if not impossible, given the massive number of changes that are known to take place during RBC storage, and present
a substantial challenge even in tractable animal models, let alone the technical and ethical challenges of human studies. Moreover, even if such experimental sophistication were achievable, it can only answer questions of what is “required” but cannot exclude what is “involved” due to issues of biological redundancy. Indeed, the lack of clear understanding that has been perpetuated by this obstacle is not limited to clearance of RBCs poststorage; rather, it seems a fair statement that discrete answers to the question of how RBCs are cleared in vivo as a part of normal RBC senescence remain undetermined. As with RBC storage, multiple pathways have been proposed and demonstrated as being possible, but identification of those that are clearly involved and/or essential has been elusive.

**Diverse conditions, variable practice, and changing landscapes**

An additional concern to the issue of sorting out the storage lesion and its effects on RBC efficacy and/or toxicity is the large number of approved RBC products and the myriad medical conditions for which stored RBCs are transfused. RBCs may be anticoagulated in CPD and CP2D (and for apheresis in ACD); units may also be supplemented with a variety of additive solutions (eg, SAGM, ADSOL-1, ADSOL-3, ADSOL-5, ADSOL-7, [SOLX], etc); the different components of these solutions have been recently reviewed. However, in general, the solutions differ in concentrations of NaCl, NaHCO₃, Na₂HPO₄, citric acid, sodium citrate, adenine, guanosine, glucose, and mannitol. Moreover, RBC units may be leukoreduced or not, depending upon country and region. Finally, there are additional prestorage variables (eg, time at room temperature after collection, irradiation, apheresis vs whole blood, etc) that have not been included in many studies, but for which there is a good faith basis to suggest a role in biology. Indeed, differences in storage solutions in Europe and Canada vs the United States have been posited as a potential cause for different results of analysis of patient outcomes as a function of transfusing stored RBCs. Perhaps ever more confounding is the wide variety of medical indications for which RBCs are given, and how the different, and at times opposing, pathophysiologies may confound observations of RBC efficacy. For instance, it has been observed that stored RBCs develop a “procoagulant activity.” If such activity correlates to the promotion of clotting in vivo, then such procoagulant activity may simultaneously be therapeutic to a patient suffering hemorrhage and lethal to a patient suffering thrombosis. This is both a concern as far as adjusting medical practice to individual patient needs, but also for the validity of clinical trials in which patients with diseases of different pathologies are included together in groups assessing medical outcomes. In the above context, the general overarching question as stated, “Is older blood bad for you?”, appears to be oversimplified to the point of being neither testable nor applicable, and more focused questions must be asked.

**Potential future applications of increased understanding of RBC storage biology**

In the field of blood product collection and transfusion, the notion of “process control” is well established and in place to variable degrees in different settings. That is to say that there is an attempt to ensure that all products are collected and distributed within the context of certain controlled parameters. In other words, blood products are all collected and treated the same, and attempts are made to sample a small number of units, from which one extrapolates a general understanding of the quality of a large number of products, which are not themselves directly tested. However, neither the scientific nor technical notion of “product control” has yet been developed or implemented. That is to say, release criteria from the blood bank (for individual units) is limited to testing negative for select pathogens, the recipient not having a detectable alloantibody against the donor unit, the unit meeting process control, and passing a visual inspection.

Of the panoply of changes that RBCs are known to undergo during storage, none is used as a quality control measure at the time of RBC release, on a unit-per-unit basis. As explained in this article, this is in large part because the field has yet to identify storage lesion measures known to predict efficacy or toxicity. However, should such factors be described, then the application to individual units is not necessarily farfetched; in the recent past (prior to electronic cross-match), essentially every unit that was transfused was cross-matched to the individual recipient.

**Summary**

Thus, how are we to answer the question of “What are the established and theoretical factors to consider in assessing the red cell storage lesion?” As detailed in this article and as reviewed extensively elsewhere, there is a well-defined list of factors that are traditional measures of the storage lesion, and a panoply of new and evolving measures, as the omics era makes its way to RBC storage. As of yet, no components of the storage lesion have been identified that adequately or accurately predict 24-hour recoveries. Moreover, although RBC circulation remains a necessary property, it is unclear that 24-hour recoveries predict efficacy beyond eliminating lack thereof due to nonviable RBCs. Because some metrics can predict blood that has lost its integrity, in vitro measures remain a guiding factor in development and refinement of new storage solutions. Nevertheless, the lack of an in vitro metric known to correlate strongly with how well RBCs will circulate posttransfusion, and the lack of knowledge as to whether 24-hour recoveries correlate to medical outcomes, remains a major obstacle. The field has access to the ability to carry out human trials and studies, use animal models, and harness systems biology of RBC storage. Future refinement of methodologies and metrics in the field will necessitate combining these approaches with a distinct focus on identifying the changes that correlate and/or are causally associated with increased efficacy and decrease in untoward effects, with a mindful consideration of different patient characteristics and clinical situations. The identification of distinct components of the storage lesion with such predictive qualities will not only guide clinical care, but also provide a rational basis for rapid storage system development and refinement. In the meantime, the lack of such metrics remains a major challenge to blood storage research.

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