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Proceedings of the Food and Drug Administration's Public Workshop on New Red Blood Cell Product Regulatory Science 2016

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Introduction

The aim of this workshop was to summarize what is known about RBC through new methods in molecular biology, genetics and physical sciences that could open new avenues for investigation on how changes in various RBC parameters brought about by storage or new processing could affect patient outcomes. The evaluation process of RBC for transfusion has not changed much over the last 20 years mainly because there has not been a strong incentive for a change. The benefits of RBC transfusions when administered in proper dose have not been questioned and, except for transfusion transmitted diseases, there have not been clinical signals that one type of a RBC is associated with more adverse events than another. The assumption has been that the relatively simple tests we are using to qualify RBC are sufficient to assure safety and efficacy of transfusions. This perspective changed almost overnight when a retrospective study linked older RBC with poorer clinical outcomes [1]. This report prompted a revival of interest in the state of the RBC at the time of transfusion and raised questions about what changes take place during storage that could mediate adverse events in the transfused patient. At the time of this conference, there were 13 randomized trials of >5,000 patients that evaluated fresher vs. older blood in patients in varying clinical settings including intensive care in adults and children, cardiac surgery, and gastrointestinal bleeding. None of these trials suggested that fresher blood is superior to older blood. Based on these data, the AABB recently published transfusion guidelines with one of the recommendations stating "patients, including neonates requiring transfusions, receive standard issue rather than fresher (storage: <10 days) RBC units (strong recommendation, moderate quality evidence)." [2] Subsequent to this conference, a landmark study was published in the NEJM (The Informing Fresh versus Old RBC

The US Food and Drug Administration (FDA) held a workshop on red blood cell (RBC) product regulatory science on 6–7 October, 2016 at the Natcher Conference Center on the NIH Campus in Bethesda, Maryland.

A full record of the proceedings is available on the FDA website (http://www.fda.gov/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/ucm507890.htm).

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Management (INFORM) Trial which showed no difference in mortality between patients transfused with fresher compared to older blood, in a pragmatic trial of over 30,000 patients in all clinical settings [3]. The vast amount of evidence now available through randomized, prospective clinical trials demonstrates no advantage to patients receiving fresher blood. The nagging question of whether blood nearing the end of its permissible storage (currently 42 days for most formulations) may result in adverse effects for transfusion recipients, however, remains unanswered. Ongoing studies in animals receiving older blood [4], human study subjects receiving older blood, which may lead to infectious complications due to iron overload [5], and retrospective studies in patients who only received blood stored for more than 35 days [6] remain a concern. Application of new methods to characterize the red cell storage lesion and development of new animal models for testing of hypotheses of connections between RBC quality and clinical outcomes will help in development of future transfusion products and in assessment of current products.

The summaries of speaker's presentations and discussions provided herein represent the basic concepts that can lay groundwork for more advanced and in-depth analysis of blood quality. Existing techniques routinely used in various facets of basic biomedical science are discussed with applicability toward translating both in vitro and novel animal model data to clinical efficacy. Evaluation of the ex vivo temporal changes in the RBC metabolome and proteome, regulation of oxygen homeostatic mechanisms and direct measurement of tissue pO_2 represent novel concepts to better understand the quality of the blood we transfuse. Similarly, advancements in understanding of the mediators (e.g. iron, hemoglobin, hemin, microparticles and immune modulators) that limit and impede effective blood transfusion are becoming more thoroughly understood. The concepts presented at this workshop are particularly relevant to better understanding changes in blood approaching later times of storage (i.e. > 35 days), effects of novel blood storage technologies and pathogen reduction systems as well as stem cell derived red blood cells and thus provide a basis for further scientific discussion of a systematic approach to assessing blood quality.

Session 1: Introduction and Background

Influence of Transfused RBC Physiology upon Recipient Oxygen Delivery Homeostasis- Alan Doctor, MD

Red blood cell (RBC) transfusion is indicated to improve O_2 delivery, or to relieve stress imposed by compensated anemia. In this context, the benefit anticipated from improving O_2 carrying capacity must be balanced against (newly appreciated, paradoxical) adverse influence of transfusion upon O_2 delivery. With improved understanding of vascular signaling by RBCs [7–9] and of the full array of defects comprising the RBC 'storage lesion', it is now understood that: (1) donor and recipient RBCs do not exhibit similar physiology and (2) RBC transfusion may cause harm (beyond transfusion reactions and transmission of infection), refered to as non-infectious serious hazards of transfusion, (NISHOT) [10]. Stored RBCs demonstrate: impaired energy metabolism/antioxidant systems, reduced deformability and increased aggregation/adhesión [11]. Moreover, stored RBCs (and unit supernatants) [12] may paradoxically impair physiologic reflexes fundamental to O_2 delivery homeostasis [13, 14] and, specifically, stored RBCs lose

hypoxia-responsive modulation of NO bioavailability (and of other vasoactive effectors such as adenosine and epoxides). This impairment undermines hypoxic vasodilation, a RBC-based reflex essential to O_2 delivery homeostasis [13–15].

Overall, storage-associated changes correspond to reduced RBC access to tissues and a loss of vascular routing reflexes essential to efficient blood flow. This consideration is relevant to bedside decision making, to clinical trial design and to evaluation of RBC products, which should include development of suitable models that quantify the relationship between approaches to processing and storage and O_2 delivery physiology (e.g. evaluating therapeutic efficacy, with respect to above indication(s)).

Insights into RBC Quality, A Century of Analysis -James C. Zimring, MD, PhD

The RBC storage lesion has been appreciated for decades as a collection of biological changes that accumulate during RBC storage. However, of the thousands of changes that have now been reported, it is unclear which changes correlate with biological function, and which are incidental. As such, while a multitude of in vitro parameters are currently measured and required as part of licensing, it is understood that no in vitro test described thus far has good predictive power as to how well RBCs will circulate and/or function post-transfusion. Some in vitro tests can rule out a good unit, however when stored RBCs are infused and circulation is measured, there remains a large variability of post-transfusion circulation even amongst units that pass all of the existing in vitro tests. Part of the historical difficulty in isolating predictive variables is a poor theoretical understanding of why senescent RBCs clear. There are multiple theories including changes in surface molecule expression, alterations in deformability, and programmed cell death (eryptosis in the case of RBCs). However, it is unclear which of these theories is correct, if any are correct, or if multiple are correct (representing redundant pathways). In vivo recoveries have been heralded as the gold standard, and the ability of an RBC to circulate seems to be a requirement for oxygen delivery. An alternative to the in vivo recovery approach as a measure of RBC quality could be elevation of bilirubin in a transfusion recipient, as indicated in a recent report on autologous units of RBCs stored and transfused to healthy volunteers after 1 to 6 weeks of storage [16]. Bilirubin reflects metabolism of removed RBCs by macrophages and it correlated with increased storage age of blood better than Chromium ^{51} labelled cells. Nevertheless, one must be mindful that circulation is a necessary (but not sufficient condition) and that the ability to circulate does not necessitate the ability to deliver oxygen. As such, even the gold standard is subject to error, and comparative efficacy of stored RBCs must always be kept in mind. Future efforts at isolating which components of the storage lesion are mechanistically involved in efficacy and/or predictive of function will be necessary for optimum progress.

Session 2: Determination of Suitability of Red Blood Cells for Transfusion

FDA Regulation of Red Blood Cell Products- Jaro Vostal, MD, PhD

The FDA reviews a broad range of RBC products associated with devices, processing and storage solutions and manufactured alternatives to donor cells. These products are subject to different regulatory pathways, but the evaluation of the RBC follows a common path.

The extent of the evaluation process for RBC products depends on how different the new product is from a conventional RBC[17]. The evaluation begins with in vitro studies to examine changes in biochemical and physical properties such as ATP, 2,3-DPG, lactate, morphology and extent of hemolysis. The next level of testing involves determination of RBC kinetics in healthy volunteers after the cells have been processed and stored. These studies are referred to as radiolabelling studies because donor RBC are tracked after infusion using a radioactive tracer. The FDA standard for radiolabelling studies is in vivo recovery of >75% of infused cells at 24 hours post infusion in 24 healthy volunteers [18]. For highly modified RBC products the evaluation also includes determination of in vivo survival and additional Phase II or Phase III clinical studies to evaluate safety and efficacy in specific patient populations. For some products it will be difficult to evaluate their safety even in Phase III clinical trials and additional monitoring through Phase IV studies will be needed in the general patient population after the product is approved.

While the FDA process for evaluation of RBC has been consistent and useful for evaluation of conventional red cells it will need to evolve to embrace new testing technologies and to be ready for evaluation of innovative and novel RBC products.

Clinical Use of RBC for Transfusion - Epidemiology of RBC Use- John R. Hess, MD, MPH, FACP, FAAAS

14 million units of RBC are transfused in the US per year or approximately 35,000 units per day. The capacity of the national blood system is 2–3 days of reserve red cells available for transfusion to cover emergency contingencies.

The use of RBC for transfusion has declined by 12% between 2011 and 2013 and this trend is continuing. This decline has been unexpected since there was a general concern in the transfusion community that an ageing US population would need more transfusion products. About half of the RBC in US are used by patients over 65 years and their numbers were expected to double between 2000 and 2025. However, there have been changes in the way we transfuse blood. Guidelines from the American Society of Anesthesiologists [19] and evidence from the TRIGGER trial recommend that transfusion triggers of 6 gm Hgb for healthy adults and 7 gm for hospitalized adults are appropriate [20]. Similarly, changes in massive transfusion protocols with early transfusion of plasma and platelets have reduced the use of RBC [21] (Glue Grant, NIH). The military has unique transfusion product needs that often lead to high rates of product expiration. New transfusion products with extended shelf lives are needed to minimize the outdating without compromise in safety and effectiveness. Development of such products should be supported and not hindered by regulations.

Evaluation of RBC Products for Transfusion-Harvey G. Klein, MD

The goals of transfusion medicine for RBC are to maximize efficacy and availability and minimize toxicity. The concept of transfusion started with a direct vascular anastomosis that attached the donor to the recipient for direct blood transfer and matured into blood banking that allowed separation of the donor and recipient by time and space. However, with banked

blood came the storage lesion which is a collective term for progressive loss of RBC quality that impacts its efficacy and safety.

Red blood cell efficacy encompasses O_2 delivery, CO_2 removal, NO binding, cytokine binding and likely other not yet identified functions. Our current assessment of RBC efficacy is based on in vitro tests and determination of in vivo radiolabeled red cell recovery post transfusion. Problems with the current evaluation process include a reliance on a limited group of healthy volunteers to evaluate the RBC with radiolabelling tests, while knowing there is substantial donor to donor variability for RBC recovery and survival and variability among donors in the storability of their RBC. However, the current evaluation process has served us well and even though it has flaws, any changes to improve it should be based on evidence. Assays to evaluate RBC should be reproducible and statistical criteria should be achievable.

Predictive Clinical Value of In Vitro Measures of RBC Quality-Jason Acker, MBA, PhD

A surprisingly high number of factors affect the state of the RBC during collection, processing and storage such that not all RBC products have the same level of quality. An important parameter of RBC quality is hemolysis. It is the final step in the RBC storage lesion and has been built into a quality standard which stipulates that hemolysis should be < 0.8% in Canada and Europe. A recent paper described the effect of collection methods on the level of hemolysis after 42 days of storage and pointed out that RBC collected by apheresis also had elevated hemolysis compared to whole blood derived RBC [22]. Donors also contribute to the variability in hemolysis after storage with female donors producing lower levels of hemolysis than their male counterparts. Another important factor in assessing hemolysis is the measurement method. A comparison of the manual and automatic methods in the same RBC found that the detection method can influence, up to 50%, the level of hemolysis reported [23].

A recent study looked at the effects of RBC processing on clinical outcomes from 91,000 transfusions to 23,000 patients and found an increase in mortality with transfusion of fresh RBC produced by whole blood method compared to other methods and older RBC [24]. This was surprising especially with concerns about stored blood. A similar study with almost 190,000 transfusions with 30,000 patients reported that receiving blood from a young donor (17–30 years) or a female donor was associated with increased risk of mortality [25]. It is important to point out that these are only associations and that mechanisms are unclear.

The methods we use for evaluation of RBC, including the radiolabelling studies, do not take into account the variables discussed above. Our goal should be to optimize the RBC characteristics, based on the donor and the effects of particular processing, to match the needs of the specific patient being transfused. We need new methods that take into account all RBC factors and have predictive capabilities of the benefits to patients.

Session 3: Methods for the Detection of RBC Processing and Storage Lesions

Omics of RBC Storage Lesions (Proteomics, Metabolomics, microRNAs) - Angelo D'Alessandro, PhD

"Omics" technologies have markedly advanced the understanding of the RBC storage lesion. Broad surveys of proteins, small molecules, and genetic loci have revealed insights into biochemical pathway alterations that can be exploited to improve RBC quality [26] [27]. RBC consume glucose during storage to maintain adequate energy states, but over time their internal energy stores are reduced and ATP and2,3 DPG decline. Declining 2,3 DPG levels may release more oxygen, contributing to internal oxidative stress and buildup of reactive oxygen species. Antioxidants such as glutathione (GSH) also decline during storage, reducing the ability to counterbalance increased oxidative stress. These changes lead to lipid and protein oxidation, protein fragmentation, and band 3 clustering in the plasma membrane. Glycolytic enzyme oxidation shunts energy metabolism through the pentose phosphate pathway at the expense of ATP production [13]. RBCs storage is improved in anaerobic conditions, resulting in less methemoglobin and higher levels of DPG and ATP. Anaerobic storage also results in higher levels of GSH, lower hemolysis, and increase resistance to hypo-osmotic stress.

Systems Biology of RBC Storage Lesions-Bernhard Palsson, PhD

From classic biochemical studies it has long been known that a wide array of biochemical and physiological parameters are altered during RBC storage. Systems biology approaches have now greatly magnified the picture revealing a 3-phase profile of metabolic alterations occurring in storage days 1–10, 10–18, and 19–42 [28]. Key metabolic changes are associated with a rise in ATP concentrations during phase 1 followed by plateau in phase 2 and decline in phase 3. Biomarkers of these storage phases have been identified and can be applied to studies of modifications of storage conditions such as temperature, and the addition of metabolites such as adenine, alternate sugars, or amino acids. Studies reveal that the metabolic shift observed later in storage is not due to an absence of adenine, and that addition of fructose or mannose can negatively impact RBC metabolism by reducing ATP and 2,3 DPG. The 3 phases of RBC biochemical change during storage provide a framework for understanding the dynamics of RBC storage and can be used in designing the next generation of storage solutions.

Genetics of RBC Storage Studies-Studies of Twins-Thomas J. Raife, MD

The heritability of energy metabolism in RBCs was established in the 1960s. In twin studies, we confirmed the heritability of pre- and post-storage RBC ATP concentrations, glutathione metabolism, hemolysis, and activity of the glycolytic pathway [29–31]. Two heritable pathways affecting RBC storage have emerged: 1) Inheritance of higher glycolytic activity correlates with higher ATP during storage, and 2) inheritance of higher concentrations of carbonic anhydrase 1 (CA1) correlate with lower ATP during storage. CA1 concentrations correlate negatively with pH, suggesting that the effect of CA1 may involve the production of acid, with resulting inhibition of glycolytic enzymes. These observations suggest a model

of blood donor variability affecting RBC storage in which inheritance of a high glycolytic rate results in higher production of ATP and better storage, whereas inheritance of higher concentrations of CA1 suppresses glycolysis and ATP production, and results in poorer storage. The data indicate that levels of CA1 and glycolytic activity are independently inherited so that combinations most favorable, least favorable, or of intermediate effect on RBC storage, are possible.

REDS-III RBC-Omics Study-NHLBI REDS Recipient Epidemiology and Donor Evaluation Study. Michael P. Busch, MD, PhD

Iron deficiency anemia and multiple inherited genetic traits in donors modulate resistance of RBCs to oxidative and osmotic stress, increase phosphatidyl serine exposure, reduce deformability, and increase splenic clearance. Other important properties of stored RBCs include immunomodulation, iron release, and blood flow. In an effort to evaluate factors affecting RBC storage, the REDS-III RBC-Omics study is developing a specimen bank and database to identify genetic influences on RBC storage and blood donors' iron physiology [32]. Spontaneous, oxidative-, and osmotic-stress induced hemolysis was measured after 42 days of storage from 14,000 leukoreduced RBC products. A genome wide association study (GWAS) was performed on DNA from leukocytes derived from leukoreduction filters using a specially developed Transfusion Medicine Array to identify genetic loci associated with high and low hemolysis and with the maintenance of iron levels in frequent blood donors. Donors with extreme phenotypes of high and low hemolysis were targeted for recall to confirm heritability and characterize the kinetics through storage of hemolysis findings in RBC components; longitudinal samples from the recall units were processed for metabolomic studies through the course of storage. Initial analyses revealed influences of gender, age, genetic ancestry and prior donation intensity on hemolysis. The GWAS analyses are in progress and metabolic pathways potentially related to properties of stored blood will also be evaluated. Additionally, the domestic component of the REDS-III program includes four research blood centers and affiliated hospitals where transfused components and associated recipient outcomes are tracked; this will permit the REDS-III donor-recipient database team to evaluate laboratory and clinical outcomes among ~20,000 recipients who received RBC components from the donors who enrolled in the RBC-Omics study. The Transfusion Medicine Array has also been applied to DNA samples from over 2,800 sickle cell patients in the Brazilian REDS-III program and to identify genetic determinants of multiple sickle cell phenotypes and complications including RBC alloimmunization.

Session 4a: Animal Models of Oxygen Delivery

Biomarkers of Transfusion Efficacy and Safety-Paul Buehler, PharmD, PhD

Demonstration of RBC transfusion efficacy and safety in animal models can provide useful proof of concept. Oxygen delivery to tissues, disease progression or mortality in specific conditions such as shock, trauma and infection represent potential for human efficacy and safety estimations.

Safety is additionally evaluated from dose dependent changes in clinical chemistry, hematology, end organ function, histology and effects on physiologic parameters such as

blood flow, blood pressure, heart rate and respiratory rate. The choice of animal species should be carefully considered because different species have particular commonalities or differences from humans.

Biomarkers particular to transfusion related efficacy and safety may include endpoints such as tissue perfusion monitored by tissue oxygenation and blood flow. For example, tissue oxygen sensing can be assessed by measuring levels of HIF-1a, an oxygen responsive transcription modifier that regulates production of hypoxia inducible genes. Coupled with oxygen level detection by the hypoxia sensitive probe pimonidazole, and regional blood flow measurements, tissue perfusion can readily be evaluated.

These types of approaches to assessing RBC transfusion in animal studies allows for more in-depth mechanistic studies not always possible in humans.

Humanized Mouse Models of Transfusion- Tim J. McMahon, MD, PhD

Either moderate or severe anemia is a strong adverse risk factor in a number of disease settings, but there is a striking lack of benefit for RBC transfusion for moderate anemia. The refinement of mouse models of transfusion has provided important new insights into the risks and benefits of RBC transfusion for anemia. Anemia itself can be modeled reliably, for example with thalassemic mice (chronic model) or with isovolemic hemodilution (acute model). Clinically relevant indices of both blood oxygen uptake and oxygen delivery can be measured in mice.

A nude, athymic mouse model, can be transfused with *human* RBC. Fresh and conventionally processed human RBC are well tolerated by mice with no adverse sequelae. However, RBC stored for weeks induce decreases in blood oxygen content, and are adherent to endothelium after transfusion. Of note, human RBCs are slightly larger than mouse RBCs. Therefore the study of key phenotypes, mechanisms or therapeutic effects, will sometimes need to be replicated in a mouse-to-mouse transfusion model. In addition, immune and other signaling molecules can vary between these two species (and by mouse strain), and this should be considered when interpreting findings. Storage lesions of both kinds - the loss of adaptive molecules or cell properties, and the accumulation of toxic mediators - can be modelled in mice. The establishment of reproducible and relevant transfusion platforms in mice means that a broad array of genetic models can be brought to bear on the important health questions raised at this conference and beyond.

Work is also under way to develop and test the clinical utility of the monitoring found useful in successfully transfused mice. For example, noninvasive microangiographic imaging, tracking actual RBC function where it happens, could be tested for its ability to inform either product development, or decision-making in anemic or transfused humans at the point of care.

Cardiovascular Effects of Blood Transfusion in a Hamster Model- Marcos Intaglietta, PhD

Transfusion of 1–2 units of blood is routinely used to treat reductions of blood oxygen carrying capacity (CaO_2) to remedy anemia and improve patient conditions, assuming that increased CaO_2 leads to increased oxygen delivery (DO_2). However mathematical modelling

of an inelastic arterial circulation with hypervolemic introduction of packed RBCs, shows that this occurs only when hemoglobin (Hb) ranges between 0–6 g/dl. Correcting anemias when Hb is > 6 g/dl lowers DO_2 because the increased hematocrit (Hct) increases viscosity and lowers flow since blood viscosity is a quadratic function of Hct. We studied the effects of transfusing 0.25, 0.5, 1.0 and 2.0 units of packed fresh hamsters RBCs (pRBC), to hamsters with Hct reductions to by ~55% using hemodilution with 5% human serum albumin 24 hours before transfusion. The hypervolemic introduction of homologous (from a donor hamster) pRBCs into the circulation of conscious anemic hamsters caused a 40–50% increase in cardiac output (CO) that was abolished by the simultaneous administration of the anti-inflammatory agent dexamethasone. This result suggests that the beneficial effects of blood transfusion are primarily a consequence of inflammatory reactions due to the introduction, or re-introduction of blood into the circulation. This hypothesis is tentatively corroborated by the increased concentration of inflammation cytokines IL-6, IL-10 and TNF-α at 5×, 10× and 3×, respectively, 2 hours after transfusion. Pilot studies with hamster blood stored 14 days (equivalent to ~ 42 days human blood storage), show that while the overall average result is the same, the variability is significantly greater, yielding both zero and 100% increases in CO. Our hypothesis requires verification of the CO phenomenon in human studies. However, it is well established that blood transfusion is an independent predictor of the systemic inflammatory response syndrome in patients being transfused with blood, and that this syndrome can be associated with the increase in CO.

Measuring the Effectiveness of Therapy by Measuring Oxygenation of the Target Tissues - Harold M. Swartz, MD, PhD, MSPH

The decision to provide RBC transfusion to patients is usually based on the need to provide adequate oxygenation and thus adequate evaluation of the utility of RBC preparations should include direct demonstration of their effectiveness in improving oxygenation in the target tissues in both preclinical studies and directly in human subjects. It is not sufficient to measure the oxygen content in the circulation because that does not account for potential barriers to delivery and consumption of oxygen in the tissues. A newly available technique, electron paramagnetic resonance (EPR) oximetry can make direct and repeated measurements of tissue oxygen in preclinical models and directly in human subjects. This method involves a one-time minimally invasive injection of appropriate paramagnetic materials into the tissues of interest. Subsequently the measurements of oxygen can be made non-invasively as frequently as desired including continuous measurements for whatever interval is desired.

Extensive successful use of this technique has been made in preclinical studies in animals ranging from mice to pigs and in essentially all tissues for more than 20 years. In the last few years the measurements have been extended to human subjects, with EPR dosimetry and oximetry measurements made in human subjects

EPR oximetry could be used to monitor oxygen in subjects receiving RBC transfusion. The appropriate evaluation of effectiveness and safety of the RBC preparations should include subjects that are representative of the population for which the therapy will be applied.

Session 4b Shock/Trauma Resuscitation

Swine Models for Shock/Trauma Resuscitation Research- Michael A. Dubick, PhD, FCCM,FACN

Large animal models have been used to model resuscitation efforts in the field and are particularly relevant to the military. The goal for design of these models is to mimic human trauma in terms of extent of hemorrhage, injury severity and the coagulation status. The models should be severe enough to have a potential for high percentage lethality, have a quantifiable blood loss and be reproducible. In general a blood loss of ≥ 50% of blood volume has been associated with high percentage mortality.

The military's focus is on the initial resuscitation to maximize short term survival. These models can be used to study the impact of resuscitations with various fluids including crystalloids, colloids, whole blood and blood components in different ratios. Common endpoints are mortality, blood loss, fluid requirements to maintain blood pressure, normalized coagulation values and inflammatory markers. A model that has been frequently used for trauma and shock situations have been immature female Yorkshire or male Sinclair mini pigs in the 30–50 kg range. Specific model selection depends on the type of research question being asked and can be carried out in sedated animals undergoing controlled bleeding or uncontrolled bleeding due to liver laceration and in combination with a blast type injury. Resuscitation with different fluids and replacement strategies can be compared to optimize endpoints such as the area under the cardiac index curve, plasma lactate, oxygen debt and survival.

Large animal models are useful for gross evaluation of resuscitation fluids and drugs but may not be sensitive enough to see dose response effects. Standardized procedures that reproduce critical care practice can be used to minimize variability across multiple centers. Polytrauma models require anesthetized animals and care should be taken when selecting anesthesia that has the least effect on hemodynamic responses.

Non-Human Primate Transfusion Models- Sylvain Cardin, PhD

Old World Monkeys (OWM), which include rhesus macaques and baboons, are phylogenically close to humans and share homology in clotting and blood proteins with humans. Physiology of OWM is also similar to humans in that they do not have splenic congestion during shock and have similar compliment activation. Human transfusion products are better tolerated by rhesus macaques as compared to swine, which mount an immune reaction to human plasma.

Macaques were used to establish a model of trauma and shock, but it was found that they were able to tolerate and compensate for hemorrhage, and hypotension in our model of shock/trauma. To reduce the effects of compensation additional blood draws were initiated when the mean arterial pressure increased by 25% and this maintained the effect of shock conditions. Resuscitation, after decompensated shock produced metabolic and coagulation changes similar to those found in human shock/trauma. Macaques are providing helpful insight into resuscitation strategies, however, working with non-human primates is complex due to specialized housing, care, stringent ethical oversight and cost. A single animal

requires $6–9,000 and an entire experiment, including husbandry, requires $25,000–40,000 per animal. These costs can approach the equivalent of a clinical trial, however, the long term benefit is that the data is translatable to human shock/trauma setting.

Session 5: Potential Mechanisms of RBC Transfusion Associated Toxicity

Toxicities of Acellular and Cellular Hemoglobin – Abdu I. Alayash, PhD, DSc

Oxidative pathways of cell free hemoglobin (CFH) and cellular hemoglobin (Hb) may relate to blood storage conditions, the so called “storage lesions”. Among the well-established pathophysiological consequences of RBC storage lesions, are formation of microparticles and release of CFH due to hemolysis that can adversely affect blood flow. Studies on Hb-based oxygen therapeutics (HBOCs) and RBC membrane microparticles provided much needed understanding of the interplay between Hb oxidative pathways and the vascular system.

Hb undergoes oxidation, where the oxygen-bound ferrous (Fe^{2+}) heme iron atom spontaneously oxidizes to the ferric/ metHb (Fe^{3+}) state (auto-oxidation). This is associated with globin instability due to generation of hydrogen peroxide (H_2O_2) resulting from the initial dismutation of superoxide products. In the presence of excess H_2O_2, the pseudoperoxidase cycle of Hb proceeds with three distinct steps: (1) initial oxidation of $HbFe^{2+}$ to a higher oxidation ferryl Hb ($HbFe^{4+}$), (2) autoreduction of the ferryl intermediate to $HbFe^{3+}$, and (3) reaction of $HbFe^{3+}$(metHb) with an additional H_2O_2 molecule to regenerate the ferryl intermediate/ferryl protein radical ($^{\cdot}HbFe^{4+}=O$). This radical may migrate and further damage the protein. These oxidative pathways can contribute to the overall toxicity associated with the use of HBOCs (acellular) which have failed to meet FDA’s safety requirements, in spite of the active research and development efforts carried out by industry over the last few decades [33].

Microparticles provide another means by which damaged and highly oxidizing Hb and heme are delivered to the vascular system. Understanding of intra- and extra-cellular Hb oxidization will shed light on the potential contribution of these oxidative side reactions to the overall toxicity associated with stored blood.

Role of Nitric Oxide and Free Heme in Mediating RBC Transfusion Toxicity – Rakesh P. Patel, PhD

Stored RBC toxicity is associated with NO depletion from extra-cellular hemoglobin and free heme-dependent activation of inflammation, oxidative stress and impairment of bacterial clearance. These mechanisms were highlighted using a mouse model of trauma-hemorrhage followed by shock and fresh or stored RBC resuscitation. A key role for free heme was indicated by hemopexin preventing acute lung injury, and decreasing the severity of instilled *P. aeruginosa* induced pneumonia in mice transfused with stored RBC. These models also demonstrated a RBC dose and storage age effect [34] which also support retrospective analyses of clinical data, the majority of which show positive associations between the age of the RBC and the number of units transfused, and adverse outcomes in trauma-hemorrhage patients [35]. Newer retrospective data focusing on massively transfused

trauma patients were also presented and showed even stronger associations. Finally, additive or synergistic effects between heme and other components in stored RBC (e.g. Hb) was discussed as an important area for future investigation and understanding of toxicities associated with trauma-hemorrhage.

Effect of Transfused RBC Storage Age on Outcome in Infected Canines – Charles Natanson, MD

To elucidate whether older stored blood worsened outcomes, as some clinical studies suggested, a meta-analysis of observational and randomized controlled trials (RCTs) was conducted. Overall, among 21 included studies, older stored blood was associated with a significantly increased risk of death (odds ratio 1.16, 95% confidence interval 1.07–1.24, p=0.0001)[36]. RCTs findings indicated that the freshest RBCs are not superior to the 2–3 week old stored RBCs transfused during current practice cannot exclude the possibility, which observational studies suggest, that older stored units, namely 4–6-week-old RBCs, increase mortality risk [37].

Two main mechanisms have been postulated to increase risks of stored blood. One proposed by Gladwin and Schecter at NIH, theorizing that increased CFH in stored blood or release due to *in vivo* hemolysis causes NO depletion, vasoconstriction and there by vascular injury. The other theory by Spitalnik and Hod at Columbia University, states that iron released from stored blood increases the risk of infections and potentially other toxicities. We found that older canine RBCs are more fragile and prone to *in vivo* hemolysis when transfused, resulting in increased release of CFH and iron. Both CFH and iron can potentially worsen outcomes in transfusion settings. CFH, as noted above, is well known to scavenge NO, an endogenous potent vasodilator, resulting in vasoconstriction, ischemia and vascular endothelial injury. Haptoglobin, the plasma protein known to bind CFH, may be totally depleted during hemolysis and unable to promote its clearance by the reticuloendothelial system (RES). In addition to CFH, hemolysis as noted above results in the release of free and protein-bound iron. Iron metabolism is ordinarily carefully controlled to prevent direct oxidative toxicity and to limit access by pathologic microorganisms. Following transfusion, transferrin and ferritin are saturated resulting in increases in free iron. Further, bacteria have evolved sophisticated strategies to scavenge iron directly from binding proteins with the help of siderophores. We investigated in canines the effects of different volumes and storage ages of transfused blood on the levels of CFH, bound iron, and free iron and potential therapeutic strategies to mitigate any harmful effects. Most of the strategies we investigated were aimed at removing or eliminating the effects of CFH and/or free iron [4, 38–41].

Coagulation Changes Related to RBC Transfusion – John W. Weisel, PhD

It has been assumed that RBCs mainly play a passive role in hemostasis and thrombosis, although new roles in these pathophysiological processes are being appreciated. The best-known effects of RBCs in clotting *in vivo* are rheological, involving laminar shearing plus aggregation and deformability of RBCs. Under conditions of activation or with increasing time of storage, RBC membranes are a major source of exposure of phosphatidylserine, which forms a pro-coagulant surface to generate thrombin by assembly of the pro-thrombinase complex. Additionally, during storage, activation, aging, and apoptosis of RBCs

leads to microparticle formation that may be responsible for increased incidence of deep vein thrombosis and other thrombotic conditions after transfusion. Furthermore, RBCs have specific receptors for endothelial cells, platelets and fibrin(ogen) that may be involved in the initiation or aggravation of thrombosis.

During contraction of blood clots, RBCs are trapped in the platelet-fibrin network and subsequent physical changes may be important for hemostasis and thrombosis. Contracted blood clots develop a meshwork of fibrin and platelet aggregates on the exterior of the clot and a close-packed, tessellated array of compressed polyhedral RBCs within. The structure and properties of contracted clots vary depending on the relative amounts of platelets, fibrinogen and RBCs and the conditions of clotting. Such close-packed arrays of polyhedral erythrocytes, or polyhedrocytes, have also been observed in human arterial and especially venous thrombi taken from patients. The extent of clot contraction and the prevalence of polyhedrocytes may be associated with thrombosis and could be a marker of prothrombotic conditions. RBCs may perform a dual role, both helping to stem bleeding while contributing to thrombosis by forming a procoagulant surface, release of microparticles, binding to other blood components, and by the formation of a tightly packed array of RBCs in a contracted clot that blocks blood flow and resists dissolution [42].

Microparticle Mediated Toxicity – Jennifer A. Muszynski, MD

RBC products contain microparticles which generally range in size from about 300 to 400 nm. In pre-storage leukoreduced RBC products microparticles from RBC themselves, platelets, endothelial cells, and leukocytes can be detected [43–45]. RBC product-derived microparticles exhibit pro-coagulant properties and may exert toxicity via dysregulated coagulation, disordered vasoregulation, or immunomodulation [46]. For example, whole blood exposed to RBC-derived MP demonstrates enhanced thrombin generation, augmented platelet aggregation, and shortened bleeding times [46–49], all of which may be mediated by exposure of phosphatidyl serine and tissue factor on the surface of the MP[48] [47]. Free hemoglobin contained in microparticles can scavenge NO which leads to impaired vasoregulation [50]. RBC-derived MP interact with immune cells *in vitro* to induce a range of inflammatory and immunosuppressive effects [43, 51, 52]. Similar effects have also been attributed to RBC-released microRNAs or other protein-bound non-coding RNA species. Together the microparticles and microRNAs may significantly contribute to the broad range of immunomodulatory (both inflammatory and immunosuppressive) effects of RBC products [53].

Transfusion Related Immune Modulation (TRIM) Safety Issues – Philip J. Norris, MD

Over the past decades multiple articles have presented in vitro and animal data to support pro-inflammatory, anti-inflammatory, or mixed effects of blood transfusion. Relatively fewer studies have been performed in humans, and these showed about equal proportions of studies demonstrating pro- vs. anti-inflammatory effects, with about a third of the studies showing no or mixed effects of transfusion on human immune parameters such as cytokine levels or immune cell function. The major limitation of available human studies is a lack of appropriate control groups and randomization. To explore immune modulation in transfusion recipients, a consortium of researchers partnered with the clinicians performing both the Age

of Blood Evaluation study (ABLE) and the RBC Storage Duration Study (RECESS) trials designed to measure the effect of RBC storage age on transfusion recipients.

Dedicated study samples were collected from study participants to test for a variety of immunological and coagulation parameters at baseline prior to transfusion and at days 2, 6, and 28 post-transfusion. Immune parameters measured included T-cell proliferation and cytokine expression after stimulation, plasma levels of pro- and anti-inflammatory cytokines, and the amount and phenotype of extracellular vesicles (EVs). Data from the ABLE trial showed decreases in some cytokines and EV levels after transfusion. Given that all subjects in the ABLE trial were transfused, it was not possible to determine if the changes were due to transfusion or the evolution of the underlying critical illness in the study subjects. Comparison of longitudinal samples from transfused and non-transfused subjects in the RECESS trial showed that most parameters measured did not differ between transfused and non-transfused subjects, though platelet-derived EVs decreased in transfused but not non-transfused subjects at the second study time point (post-transfusion in the subjects who were transfused). These data support the measurement of immune parameters in trials of subjects who are randomized to transfusion or not, as this would be the only method to definitively define the effects of transfusion on immune and coagulation parameters.

Final Thoughts - Paul M. Ness, MD

The FDA sponsored this very timely “Pre-Clinical Evaluation of Red Blood Cells for Transfusion” workshop because of continuing concerns about whether older RBC can cause adverse reactions with increased morbidity or mortality for patients. The database created in the INFORM study might hold the key to this persistent question [54]. If the dilemma about differences in clinical impact between fresh and old RBC persists, new studies in patients might now be justified to address this specific goal. Prudent options might also include shortening the currently accepted storage period for licensed preservative solutions or adopting better preservative solutions, taking advantage of the wealth of knowledge accumulated from the many studies that have addressed the storage lesion.

The “Pre-Clinical Evaluation of Red Blood Cells for Transfusion” workshop addressed many questions and provided reassuring answers to many concerns, but several issues will certainly require continuing attention. Our current storage solutions are licensed for 42 days, but the question of whether we really need 42 days of storage in an era with new computer and transport technologies should be considered. Longer storage may continue to be critical for distant sites or military operations. Although outdating might increase along with some associated costs, these concerns must be considered if patients would benefit from shorter blood storage duration. Another concern which was not presented at the conference was whether pathogen reduction systems for RBC currently under development could worsen the clinical problems that have been associated with the storage lesion. A final issue that was raised was how the regulatory process for licensure of RBC would be affected by the many new and unique ways that can be applied to measure in vitro effects of blood storage. If the regulatory process becomes even more complicated and comprehensive than the current process, industry may decide not to develop improved blood storage solutions for fear that the expense and time commitment may never become financially supportable, resulting in a

stagnation of clinical improvements available for patient care. All of these concerns can be addressed in the future, but the recognition that tremendous progress has been made to understand the storage lesion for RBC and potential ways to mitigate it should be acknowledged with appreciation of the strong collaborative efforts of academic medicine, industry, and the government [55].

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