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Does prolonged storage of red blood cells cause harm?

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Summary

Red blood cells (RBCs) degrade progressively during the weeks of refrigerated storage. No universally accepted definition of “fresh” or “old” RBCs exists. While practices vary from country to country, preservative solutions permitting shelf life as long as 7 weeks have been licensed. Transfusion of stored RBCs, particularly those at the end of the approved shelf life, has been implicated in adverse clinical outcomes. The results of observational analyses, animal models and studies in volunteers have proved provocative, controversial and contradictory. A recently completed randomized controlled trial (RCT) in premature infants exemplifies the difficulties with moderately sized clinical studies. Several other RCTs are in progress. The effect of RBC storage may well vary according to the clinical setting. Resolution of the importance of the storage lesion may require large pragmatic clinical trials. In the meantime, institutions involved in blood collection and transfusion should explore strategies that assure blood availability, while limiting the use of the oldest RBCs currently approved by regulation.

Keywords

Red cell units; red blood cells; storage; therapy; adverse effects

Introduction

An estimated 90 million units of red blood cells (RBCs) are transfused worldwide annually. Transfusion of RBCs saves lives and enables many medical therapies. RBCs meet the definition of an essential medicine, although use became widespread before and without the rigorous evaluation of randomized clinical trials (RCTs) (Klein, 2013). Despite well-publicized, immunological and rare infectious adverse events (Vamvakas, 2007; Klein, 1999), in developed countries RBC transfusion has a therapeutic index exceeding that of many common medications (Klein *et al*, 2007). However, RBC shelf life is determined and strictly regulated not on the basis of clinical studies, but based on *in vitro* haemolysis and *in vivo* radiochromium label recovery and survival studies (Dumont & AuBuchon, 2008). The

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acceptable limits were originally derived from expert opinion, not through correlation with clinical outcome (Hess, 2012).

For almost 20 years, retrospective and prospective observational studies have hinted at a harmful effect of RBC storage (Aubron *et al*, 2013; Lelubre & Vincent, 2013). A large retrospective single-centre study comprising 6002 patients (Koch *et al*, 2008) raised both interest and concern about the quality of stored RBCs and their potential toxicity. A formal meta-analysis of observational studies and RCTs focusing on patient survival rates concluded that regardless of clinical situation, trial size or volume of blood transfused, older stored blood was associated with a significant increase in mortality (Wang *et al*, 2012). This article will review and analyse current RBC preservation issues, animal data, volunteer studies, the observational database and the several ongoing RCTs that are designed to correlate clinical outcomes with RBC storage time.

RBC shelf life in current clinical practice

For most of the 20th century, the goal of red cell preservative solution research was to extend RBC shelf life in order to enhance inventory control and reduce outdating. Most blood providers extended the RBC shelf life from 21 to 28 days with the addition of phosphate to the solution, to 35 days with the addition of adenine, and to 42 days when additive solutions became widely introduced (Hogman *et al*, 1978; Beutler & West, 1979; Hogman, 1985; Moroff *et al*, 1990; Hess, 2006). A brief survey (Table I) documents the variability in the approved versus the practiced RBC shelf life in different healthcare systems (Sparrow, 2012). The longest shelf life currently in clinical use is 49 days (Zehnder *et al*, 2008). A new additive solution has been recently Conformité Européenne (CE)-certified for 56-day storage in Europe, and Food and Drug Administration (FDA) licensed for 42-day storage in the US (Hess *et al*, 2006). Some blood centres have opted to voluntarily retain 35 or 42 days and forgo the opportunity to increase shelf life, although their national regulations allow extending the shelf life to 42 or 49 days. Japan has elected to restrict the “42-day RBC” to a 21-day shelf life since 1995; the shorter storage period reduced the risk of septic reactions from slow-growing bacteria and permitted universal irradiation of RBC to prevent transfusion-associated graft-versus-host disease (GvHD) (Sakakibara & Juji, 1986; Otani *et al*, 2012).

Some national blood systems have shortened the RBC storage interval in the expectation that the shortened shelf life improves RBC quality. The UK restricts RBC shelf life to 35 days by policy (UK Blood Transfusion & Tissue Transplantation Services, 2013) despite the use of additive solutions deemed suitable for 42 day storage by law (UK Legislation, 2005). The Netherlands elected to apply 35-day storage even with the availability of additive solutions, because inventory management is satisfactory with this shorter outdate and discard rates remain less than 2%. Other health care systems have instituted a policy of discarding RBCs, once storage has reached the last 7 days of the longest acceptable shelf life for their additive solutions. Large regions and entire nations have determined that a 35-day shelf life can be safe and cost-effective for providing RBC support.

Retrospective, prospective observational and case-control clinical studies

Meta-analysis of studies on mortality—We conducted a formal meta-analysis of studies comparing patient survival rates associated with the transfusion of fresh versus older RBCs (Wang *et al*, 2012). As no universal definition of “fresh” or “old” RBCs exists, we accepted the definitions used in the various studies. Seventeen to 387,130 patients were enrolled between 1991 and 2009 in 3 RCTs (Schulman *et al*, 2002; Fernandes da Cunha *et al*, 2005; Hebert *et al*, 2005), 6 prospective observational and 12 retrospective studies. The effects were similar across these 21 studies and overall mortality was increased significantly for patients receiving older RBCs. Based on these predominantly retrospective data, published mortality rates and odds ratios (Wang *et al*, 2012), one would have to transfuse between 97 and 69,428 patients with exclusively fresh RBCs to save one life.

Six studies enrolled trauma patients (Schulman *et al*, 2002; Murrell *et al*, 2005; Weinberg *et al*, 2008a; Weinberg *et al*, 2008b; Spinella *et al*, 2009; Weinberg *et al*, 2010), 6 cardiac surgery patients (Hebert *et al*, 2005; van de Watering *et al*, 2006; Yap *et al*, 2008; Koch *et al*, 2008; Robinson *et al*, 2010; van Straten *et al*, 2011) and 9 a mix of varied patient populations (Mynster & Nielsen, 2001; Fernandes da Cunha *et al*, 2005; Leal-Noval *et al*, 2008; van Buskirk *et al*, 2009; Gauvin *et al*, 2010; Karam *et al*, 2010; Eikelboom *et al*, 2010; Edgren *et al*, 2010; Pettila *et al*, 2011). The results of each of these 3 subgroups were consistent with an overall increase of mortality associated with older RBCs, as they were in small and large studies (<500 versus > 500 patients) and in studies of patients receiving on average 3 RBCs or less versus more than 3 RBCs per patient. Seven studies reported serious adverse events; 3 studies an overall significant increase of pneumonia and 3 others showed an overall increase in multiple-organ-dysfunction associated with transfusion of older blood (Wang *et al*, 2012), suggesting an increased risk of death from old RBCs.

Current reviews—A plethora of clinical data, primarily from retrospective studies, were recently published in reviews (Aubron *et al*, 2013; Lelubre & Vincent, 2013; van de Watering, 2013). Additional retrospective studies in different clinical situations appear almost monthly (Janz *et al*, 2013; Middelburg *et al*, 2013; Saager *et al*, 2013). Despite the numerous studies, practitioners are left with divergent results for clinically important outcomes.

Among the 32 studies tabulated by one review (Aubron *et al*, 2013), 18 reported a harmful effect of prolonged RBC storage, while 14 did not. The 7 prospective observational or case-controlled studies were evenly distributed (4 with and 3 without significant harmful effect). Of note, the 4 prospective randomized studies in this tabulation showed no significant harmful effect (Wasser *et al*, 1989; Schulman *et al*, 2002; Hebert *et al*, 2005; Kor *et al*, 2012).

One report tabulated 55 studies including 8 small RCTs (Lelubre & Vincent, 2013). The 47 non-RCT studies addressed clinical outcomes, including mortality (22 studies); occurrence of infection (18); organ failure (12); tissue oxygenation and microcirculation (11); length of hospital stay (9) and other outcomes (8). The considerable heterogeneity and methodological flaws of the included studies prevented the authors from combining the data or trying to determine the reasons for the different effects (Lelubre & Vincent, 2013).

The largest retrospective cohort comprised 364,037 patients, and concluded that the excess mortality, if any, caused by older RBC is probably less than 5% (Edgren *et al*, 2010). Among the 4 next largest studies, 2 studies comprising 6002 (Koch *et al*, 2008) and 4933 patients (Eikelboom *et al*, 2010) reported a harmful effect, while the 2 other studies, comprising 3475 (van Straten *et al*, 2011) and 2732 patients (van de Watering *et al*, 2006), did not. Possible confounders, such as variations in leucocyte depleted RBC blood products or ABO matching, are of concern and not always controlled or even documented in detail.

The retrospective and small prospective studies raise concerns about the efficacy and toxicity of older stored RBCs. However important as they are in generating hypotheses, the data are not sufficiently robust to warrant changes in national blood policies. In retrospective studies, transfusions may represent no more than a marker of illness severity independent of other parameters, like the APACHE II (Acute Physiology and Chronic Health Evaluation II) score. The known confounding factors are difficult to adjust, no matter how much effort authors put into statistical analysis. Furthermore, publication bias favouring positive studies is an unfortunate fact. RCTs are the current “gold standard” in evidence-based medicine.

Randomized clinical trials (RCTs)

There is a dearth of reliable prospective controlled clinical data regarding the safety and effectiveness of RBC transfusion and there are surprising gaps, despite the 142 related RCTs published up to 2009 (Wilkinson *et al*, 2011). Eleven RCTs evaluated RBC storage times with a median of 35 patients (range 10 – 237) and only 2 RCTs addressed clinical outcome, such as postoperative bleeding (Wasser *et al*, 1989) and mortality in a trial published subsequently (Fergusson *et al*, 2012). Physiological parameters, rather than mortality, were the primary outcomes in most of the completed RCTs (Table II). None of these RCTs detected any effect of RBC storage time on clinical outcomes.

The first of the larger RCTs addressing mortality as the primary outcome was recently completed (Fergusson *et al*, 2012). The use of fresh RBCs compared with standard blood bank practice did not improve major neonatal morbidities, such as necrotizing enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular haemorrhage, or death, in premature, very low birth weight infants requiring a transfusion. However, the average duration of RBC storage in the standard of care group (14.6 days) does not reflect the average RBC storage in the US (18 days) and certainly not the practice of some centres that routinely store RBCs for 21 days or longer (Fernandes da Cunha *et al*, 2005). The 5 large ongoing RCTs (Table III) use mortality or multiple organ dysfunction as primary outcomes and study fresh RBC versus standard of care. The patient cohorts studied will be important for the applicability of the results and include all acute care inpatients, critically ill patients in adult intensive care units (ICUs) and patients with complex cardiac surgery. The results of these large RCTs are not expected for several years. In the meantime, standards of practice must rely on evidence from other sources.

RCT design considerations

The power of an RCT may be smaller than anticipated, if qualitative properties of RBCs differ among the participating institutions. Such variations of RBC quality, other than

storage time, may be caused by differing shelf life for identical additive solutions (Table I); different leucocyte removal procedures; the lack of leucocyte removal; gamma irradiation of RBCs or other small, seemingly innocuous variances in preparation techniques and handling procedures. Confounding factors need to be evaluated, particularly for multi-centre and multi-national RCT consortia, such as INFORM and TRANSFUSE (Table III).

An informative theoretical study (Pereira, 2013) simulated the possible outcomes of hypothetical RCTs comprising 2000 patients each. The experimental (8, 10, <14 and 14 days) and control arms (2–42, >14, >20 and 21 days) of the 4 RCTs were modelled after actual study designs (Koch *et al*, 2008; Lacroix *et al*, 2011; Steiner *et al*, 2010); NCT00458783). Five different temporal patterns linking clinical outcomes with the RBC storage lesion were examined (Fig. 1). The power of the modelled RCTs consistently exceeded 80%, a limit often deemed desirable for RCTs, for one temporal pattern only (Fig. 1, model 1). It is sobering to note that surprisingly low study powers, often below 20%, are more the rule than the exception for the other temporal patterns (Fig. 1, models 2 to 5). While these limitations may be impossible to overcome, common pitfalls can be avoided (van de Watering, 2011).

Pilot studies are conducted to assess feasibility, acceptability and blinding to aid in the design of large, pragmatic RCTs with a transfusion medicine topic, such as RBC storage (Hebert *et al*, 2005; Bennett-Guerrero *et al*, 2009; Aubron *et al*, 2012; Heddle *et al*, 2012). Patients can be randomized to a fresh RBC group, which is commonly perceived as the improved treatment arm. Patients should not be treated with old RBC by design, and the control group is typically standard-issue RBC (oldest in inventory). With the possible exception of chronic and neonatal transfusions, standard of care is “first in, first out” and the oldest available ABO identical RBC unit is transfused. This ethically imperative constraint restricts the design options and can limit the power of any RCT, because any adverse effect conveyed by the oldest RBCs only (e.g. model 2 in Fig. 1) may be missed, when such RBCs are hardly occurring in the standard of care arm.

An accepted frequent deviation from standard of care is the use of the oldest ABO compatible RBCs, which is ABO antigen compatible, but not identical, with the recipient’s ABO blood group. This deviation may affect recipients of blood group B and AB more often than those of blood group A, while recipients of blood group O, of course, can be transfused only with ABO identical RBCs. These discrepancies are not trivial and ABO blood group differences among the study arms should be carefully evaluated (Frenzel *et al*, 2008; Dzik, 2008; Middelburg *et al*, 2013) if they are unavoidable. The storage times of the standard inventory practice units would vary, depending on the ABO and Rh type and the vagaries of the blood supply (Steiner *et al*, 2010; Cheng *et al*, 2010; Middelburg *et al*, 2013).

If the combined results of RCTs should not favour fresh RBCs (or old RBCs), clinical differences may exist that are too small to be discerned within the scope of the clinical studies but still important enough to be of public health concern, for example an excess mortality of less than 5% (Edgren *et al*, 2010). The decision on an acceptable RBC storage period is unlikely to rest on RCTs alone and should include other clinical and non-clinical evidence. A comparative effectiveness research (CER) approach could be pursued,

particularly if the ongoing RCTs end inconclusively (Klein, 2012; Blajchman et al, 2012; Klein & Natanson, 2012). In contrast to double-blinded RCTs of efficacy, CER compare two different but accepted standard practices. CER studies are often referred to as large, simple pragmatic trials and should allow enrollment of large cohorts (>10,000 patients) within a short time. Our decisions should also draw on basic principles and the understanding derived from pre-clinical studies with volunteers, small and large animal studies *in vivo* studies, and *in vitro* experiments.

Pre-clinical studies with volunteers

The effect of fresh-versus-old RBCs on physiological parameters can be assessed reliably and safely by transfusing autologous RBCs to healthy volunteers (Table IV). Such studies, unlike clinical trials, permit maximal separation of the age of stored RBCs. No differences were found for cognitive and pulmonary function or hyperaemia in three different volunteer studies. The informative study by Hod *et al.* (2011) documented highly significant increases of serum iron and transferrin saturation at 4 h after transfusion of older RBCs. Ferritin concentrations increased from baseline only after transfusion of older RBCs. While non-transferrin-bound iron concentration was not significantly increased after fresh RBCs, it progressively increased until 4 h after transfusion of older RBCs. The ongoing haemolysis during RBC storage may explain the significantly increased serum total bilirubin peaking at 4 h, which correlated with a peak of unconjugated bilirubin for some volunteers and a small, but significant, rise in serum conjugated bilirubin. While the results from volunteers raise few concerns, the volumes transfused are small and the recipients healthy so the effects may be quite different in critically ill patients. The exact study details, such as RBC preparation and volumes transfused, need to be considered when extrapolating from these convincing data.

Small and large animal *in vivo* studies

Animal studies can be designed to address specific physiological questions and mechanisms that could not be done using volunteers. Such studies are particularly important for understanding the underlying mechanism, unlikely to be attained by RCTs, and eventually result in the formulation of improved RBC units. The results of these studies may contribute to the design of RCTs as much as they complement the results of RCTs. There are benefits and limitations for RBC transfusion in models (Simonova *et al.*, 2013) of small animals, such as mice (Gilson *et al.*, 2009), rat (d'Almeida *et al.*, 2000), guinea pig (Baek *et al.*, 2012) and cat (Wardrop, 1995), as well as larger animals, such as dog (Wardrop *et al.*, 1997) and sheep (Simonova *et al.*, 2013). Animal studies may be the only way to test the hypothesis that old RBCs affect outcome by a “late” temporal pattern (Fig. 1, model 2) or in few critically ill patients, such as those with severe bacterial pneumonia.

Murine—Mouse and human RBCs show a progressive decline in survival depending on storage time (Gilson *et al.*, 2009), unlike the precipitous loss of viability reported for rat RBC (d'Almeida *et al.*, 2000). Non-transferrin-bound iron concentration and acute tissue iron deposition were increased after transfusion of old mouse RBCs, which may initiate inflammation not seen after fresh mouse RBCs (Hod *et al.*, 2010). The increased non-transferrin-bound iron was implicated in enhancing bacterial growth *in vitro* (Hod *et al.*,

2010), which could not be confirmed subsequently in a prospective human study (Hod *et al*, 2011). Old mouse RBCs induced more cytokines and alloimmunization than did fresh RBCs, which may introduce another variable in RBC storage studies (Hendrickson *et al*, 2011). In a rat model, 28- and 35-day-old rat RBCs promoted lung oedema (Nicholson *et al*, 2011); no effect of rat RBC storage on survival was tested. In an *ex vivo* model with aortic rings from rats, older RBCs inhibited NO-induced vasodilation (Alexander *et al*, 2013).

Guinea pig—Transfusion of 28-day-old guinea pig RBCs led to intravascular haemolysis, hypertension, vascular injury and kidney dysfunction (Baek *et al*, 2012). This plasma haemoglobin-driven toxicity could be attenuated by infusion of haptoglobin. The precise mechanism of injury is unknown but may involve the direct toxicity of cell free haemoglobin, iron, scavenging of nitric oxide (NO) or a combination of such mechanisms (Schaer *et al*, 2013).

Dogs—The most striking results of any animal study on old RBCs so far have been observed in canines (Fig. 2): 42-day-old dog RBCs dramatically increased mortality in dogs with experimental *Staphylococcus aureus* pneumonia (Solomon *et al*, 2013). This first RCT used standard blood bank techniques and evaluated if RBCs transfused at the end of the storage period increase mortality. Canines with pneumonia were treated like critically ill patients with bacterial pneumonia in an intensive care unit. RBCs were collected and prepared by an FDA-licensed canine blood bank mimicking procedures and technologies used for human RBCs (Wardrop, 1995; Steiner *et al*, 2010). The bacterial challenge dose was optimized in this validated blinded canine model to account for the effects of multiple transfusions on mortality.

Increased systemic and pulmonary artery pressures indicated that old RBCs were more vasoactive than fresh RBCs (McLaughlin *et al*, 2013; Badesch *et al*, 2009). Pulmonary hypertension caused right ventricular dilatation and, by adversely affecting left ventricular filling, resulted in marked tachycardia to maintain cardiac output. Prolonged haemolysis of old RBCs resulted in increased cell-free haemoglobin and decreased haptoglobin. Old RBCs caused a steady rise in NO consumption capability of plasma for days (Jeffers *et al*, 2006), indicating the presence of oxyhaemoglobin, the vasoactive reduced form of haemoglobin, known to scavenge NO (Reiter *et al*, 2002; Minneci *et al*, 2005; Rother *et al*, 2005; Yu *et al*, 2008; Hu *et al*, 2010; Donadee *et al*, 2011). Prolonged exposure to oxyhaemoglobin resulted in ischaemic vascular damage at the site of tissue injury in the lung, causing gas exchange abnormalities, pulmonary arterial hypertension and an increased risk of death.

Non-transferrin-bound and labile iron, the toxic iron moiety, were elevated only during transfusion, but not associated with survival (Solomon *et al*, 2013). NO scavenging and *in vivo* haemolysis were augmented after transfusion of old RBCs, which appeared to result in an excess of non-transferrin-bound and labile iron, but worsened outcome only in the presence of an established infection (Wang *et al*, 2013). The availability of iron, circulating 8 to 12 h after bacterial challenge, may have promoted bacterial growth contributing to mortality. Washing RBCs before transfusion had a significantly different effect on canine survival, multiple organ injury and plasma iron and cell-free haemoglobin concentrations, depending on the age of stored RBCs. Washing old RBCs improved canine survival, whereas

washing seemed to damage fresh RBCs and increased canine mortality (Cortes-Puch *et al*, 2014). Studies in human volunteers, mouse, guinea pig and dog hinted consistently to significant increases in cell-free haemoglobin and iron, plausibly caused by an increased haemolysis of the old, more fragile RBCs.

Sheep—Similar to the TRALI2 study in humans (Kor *et al*, 2012), which examined transfusion-related acute lung injury (TRALI) and was expanded to study RBC storage effects, a TRALI study prompted the development of a sheep model (Tung *et al*, 2011; Simonova *et al*, 2013). Transfusion of 40-day-old autologous RBCs increased pulmonary vascular resistance and pressure, which was worsened by an NO synthase inhibitor and ameliorated by NO inhalation (Baron *et al*, 2012). These observations were compatible with the NO and free iron findings in dogs. As TRALI is thought to be caused predominantly by plasma and not by cellular components, initially the effect of the supernatant from stored human RBCs was tested in sheep (Tung *et al*, 2012). Such human supernatants decreased arterial pressure and cardiac output in lipopolysaccharide-primed sheep more than did supernatants from stored human platelet components (Tung *et al*, 2012). Ovine RBCs stored for 35 to 42 days induced pulmonary arterial hypertension but not TRALI (Fung *et al*, 2013).

It is not clear to what extent rodent, canine, sheep or, for that matter, any non-primate models can mimic the variety of human clinical circumstances, and whether canine or other RBCs are equivalent to similarly stored human RBCs (McCullough, 2013). Even if transferable, the current canine study results may apply only to severely ill patients with infections and RBCs at the very end of the shelf life permitted by the additive solutions used.

RBC storage

RBC storage lesion—The original concern regarding RBC storage time and maximal acceptable shelf life for patient care evolved from observations of the RBC storage lesion(s) (Fig. 3) (Dzik, 2008; Zubair, 2010; Blajchman *et al*, 2010; Zimring *et al*, 2011; Pavenski *et al*, 2012; Koch *et al*, 2013). Although the RBCs rest at +4 °C and are not agitated for the duration of their shelf life, RBCs are exposed to oxidative stress and undergo metabolic changes. The accumulating metabolites, altered proteins and particles, such as microvesicles, may be released into the supernatant (Klein *et al*, 2007). The damage affecting the membrane and cytoskeleton results in altered deformability and shape and compromises RBC integrity (Frank *et al*, 2013).

The dynamics of the processes vary widely: Some effects occur within a few hours of blood donation, whereas other effects represent the accumulation of changes that occur over days and weeks. As a consequence, it is conceivable that there may be examples of determinants for the RBC storage lesion, which are represented by any of the temporal patterns discussed previously. Further, donor-related biological variability should be expected in the kinetics of change as schematically shown for the 5 models (Fig. 1). The temporal pattern linking clinical outcomes with the critical determinant of the RBC storage lesion may also vary among patients and their different clinical conditions.

Data from dogs, sheep and mice support the hypotheses that NO depletion and free iron release, occurring as a consequence of the RBC storage lesion, contribute to the detrimental

effects of old RBCs (Solomon *et al*, 2013; Wang *et al*, 2013; Baron *et al*, 2012; Hod *et al*, 2010). However given the extent of the changes in the RBC, other mechanisms may well be involved.

Ex vivo and in vitro studies—⁵¹Cr-labelling, widely used to study RBC survival *in vivo* and a measure generally required by licensing authorities, has intrinsic variability in addition to the donor-to-donor biological variability (Dumont & AuBuchon, 2008). Flow cytometry allows demonstration of a nearly identical long-term RBC survival *in vivo* for fresh and old RBCs, once a removal-prone fraction in the older RBCs is eliminated (Luten *et al*, 2008). Haemoglobin increments at 48 h did not differ between fresh and old RBCs in an RCT involving 10 patients (Wallis *et al*, 2005), while 2,3-diphosphoglycerate (2,3-DPG) was significantly lower up to 48 h after transfusion of the old RBCs. No clinical efficacy studies are required or recommended for assessing RBC quality in the US and European Union (EU).

Types of RBC preparation and shelf life—Many RBC preparation and storage conditions have been studied to enhance RBC survival in the storage container, as *in vitro* changes are considered to predict clinical outcomes (Hess, 2012; Orlina & Josephson, 1969). Even within the past decade, RBC preparation and storage solutions have been modified (Greening *et al*, 2010). Although the general approach becomes standardized, many small technical variations add to the inherent complexity of the RBC blood product (Hess *et al*, 2009). As a biological product from human volunteer blood donors, its source cannot be standardized. Unlike small molecule drugs, each RBC unit is considered a “lot” or a “batch.” A substantial donor-to-donor variability in RBC storage, haemolysis and survival has been recognized since the 1960s with non-normal distribution, in that the RBCs of approximately two-thirds of the donors store better than the mean; there are donors whose RBC store exceptionally well (Hess, 2012; Dumont & AuBuchon, 2008). Biomarkers to predict suboptimal storage and to identify “poor storers” do not currently exist, but would be extremely valuable (Francis *et al*, 2013).

Whole blood is typically collected into a sterile plastic bag and anticoagulated with a defined concentration of citrate, for example a CPD (citrate, phosphate, dextrose) solution. The packed RBC are then transferred into a second bag with an “additive solution”, for which SAGM (sodium, adenine, glucose, mannitol, 376 mOsm/l) is commonly used and approved for a 42-day RBC shelf life (Hess, 2006). Approval of additive solutions depends on *in vivo* RBC recovery and survival as well as on measurement of analytes thought critical for RBC survival. The most important RBC analytes are adenosine 5'-triphosphate (ATP) concentration and 2,3-DPG recovery rate (Dumont & AuBuchon, 2008). RBCs are regulated by drug acts, biological medicine regulations (UK Legislation, 2005), pharmaceutical affairs laws and national standards (UK Blood Transfusion & Tissue Transplantation Services, 2013), which vary by country. In the UK, the evaluation of new procedures to produce RBCs is detailed (chapter 8.2, UK Blood Transfusion & Tissue Transplantation Services, 2013). Commonly accepted RBC recovery and haemolysis rates after 12 weeks storage may be achieved with non-standard solutions, which are not yet approved (Hess, 2006; Hess *et al*, 2003).

Leucocyte removal during RBC preparation is commonplace in Europe and Canada, but not standard in the US. RBC preparations differ in many regards that can be easily delineated *in vitro* and may be of clinical relevance (Bordin *et al*, 1994; Willy *et al*, 2000). Irradiation, which is used for specific indications in some institutions or universally for other hospitals and national blood supplies, is well documented to damage the RBC membrane. The plasticizer diethylhexyl phthalate (DEHP), which leaches from the polyvinyl chloride (PVC) bags that are widely used for RBCs, affects the RBC membrane stability and limits membrane loss by microvesiculation beyond 3 weeks storage (Rock *et al*, 1984; AuBuchon *et al*, 1988; Hess, 2006). While plasticizer reduces haemolysis, a critical parameter for approval of new blood container devices (Hill *et al*, 2001), possible DEHP toxicity remains a concern, particularly for premature infants (Luban *et al*, 2006).

The overall quality of a RBC blood product may actually improve during the first few days of storage. Bacterial contamination during the donation process may be cleared within the first few hours of storage of whole blood or RBC fraction, before the leucocytes are removed. Storage at +4 °C for several days may reduce the risk of spirochetes, some cell-associated viruses and mononuclear cells responsible for GvHD (Sakakibara & Juji, 1986). Whereas no claim has been made that RBC quality improves during storage beyond the first few days, an abundance of *in vitro* evidence implicates refrigerated storage as increasingly detrimental to RBC quality (Chin-Yee *et al*, 1997; Tinmouth & Chin-Yee, 2001; Solheim *et al*, 2004).

Novel RBC preservatives—Evaluation of additive solutions *in vitro* by studying the proteome indicated an aging process that was not addressed by either of the two additive solutions commonly used in North America and Europe (D'Amici *et al*, 2012). Anaerobic storage, which was shown to improve *in vivo* RBC survival in a crossover RCT involving 8 volunteers has been proposed as a novel approach (Dumont *et al*, 2009). RBC stored under argon are not exposed to the oxidative processes thought to induce structural damage; molecular iron released from haem could not catalyse reactions with oxygen as its substrate (Yoshida & Shevkopyas, 2010). Research opportunities in optimizing RBC storage, such as new additive solution, anaerobic storage or addition of free radical scavengers, have recently been summarized (Wagner *et al*, 2014).

Clinical guidelines

Guidelines in some healthcare facilities require transfusing fresher RBCs to vulnerable patient groups, including fetuses for intrauterine transfusion, premature infants, all newborns, or patients undergoing cardiac surgery. Some chronically transfused patients, such as those with thalassaemia and sickle cell disease or haematological malignancies, receive fresh RBCs to extend transfusion intervals and reduce the total RBC volume transfused with its concomitant iron burden.

In the US, the guidance by the American Association of Blood Banks (AABB) Standards (Carson, 2012) and recommendation by the AABB Technical Manual do not restrict RBC storage time for any adult patient group (Nester & AuBuchon, 2011); RBCs less than 7 days old are recommended for intrauterine transfusions (Kennedy, 2011). In the UK, RBCs must

be used by the end of day 5 for intrauterine (chapter 7.22) and exchange transfusions (chapter 7.24), and for large-volume transfusion of neonates and infants under 1 year (chapter 7.26, UK Blood Transfusion & Tissue Transplantation Services, 2013). In Germany, RBCs should be as fresh as possible, but less than 7 days old, for intrauterine transfusion and exchange transfusion of preterm babies and neonates, and must not be older than 28 days for large-volume transfusion of neonates (chapter 4.4.2) (Bundesärztekammer & Paul-Ehrlich-Institut, 2010). Although these guidelines have long traditions, seem to be intuitive and could prove to be beneficial for patients, evidence has been lacking or inconclusive that outcome is affected by the RBC storage time in any patient group.

Besides perinatal patients, no cohorts of vulnerable, critically ill patients are defined at the national levels to preferably receive fresher RBCs.

Inadvertent consequences of using fresher RBCs—A transfusion policy with fresher RBCs could curtail the blood supply in critical situations for some patients. Adverse effects of transfusion are strictly monitored, but adverse effects caused by an inadequate blood supply are notoriously difficult to gauge at the aggregate level (Flegel, 2012). Patients in critical need could be harmed by either the lack of RBCs or by availability of blood that is not ideally matched by blood group. Such a circumstance would reverse any benefit gained from slightly “fresher” RBCs for the majority of patients.

Transfusing fresher RBCs might benefit some patient cohorts, such as severely ill patients in intensive care units or patients with infections, while offering a limited or no benefit for the majority of patients. If fresher RBCs were generally transfused without restricting the RBC shelf life, older RBCs might be sequestered to certain patients, such as those with trauma and extensive surgical procedures requiring large amounts of RBCs; paradoxically, these patients might benefit most from fresher RBCs. Some neonatologists avoid transfusing the freshest RBCs by using aliquots drawn from a single donor when limiting donor and infectious disease exposure is considered the superior objective; fresh RBCs and limiting donor exposure may not be conflicting goals, if the shelf life is 28 days or shorter (Fernandes da Cunha, 2005).

A review of the reports on heavily transfused patients concluded that several studies indicate that fresh RBC might be associated with adverse outcomes (van de Watering, 2013). In a retrospective study, the same group reported an almost 2-fold increase in mortality rate after transfusion of fresh compared to older RBC (Middelburg *et al*, 2013). The proposition that RBCs with a shelf life of 5 to 21 days may have any negative effect, worse than older RBCs, is not intuitive and has not been widely shared.

Implementing and maintaining any policy of fresher RBCs may require major operational changes to the current practice of collection, storage and transfusion. Recruiting additional blood donors, increasing RBC outdateding or more complex inventory management will increase costs. For changes that involve major practical and organizational consequences, such as reduction of the RBC storage time, a multi-tiered, stepwise and carefully monitored approach seems prudent.

Conclusion

Retrospective and prospective observational studies and a meta-analysis of 21 studies reporting on mortality have suggested that fresher RBCs may benefit defined patient groups or all patients, whereas the only completed RCT failed to show improved outcomes in premature, very low-birth weight infants transfused with fresher RBCs (Fergusson *et al*, 2012). By one calculation, between 97 and 69,428 patients needed to be treated with exclusively fresh RBC to save one life (Wang *et al*, 2012). Studies in several animal models report evidence of organ toxicity and increased mortality when older RBCs are transfused. The several ongoing RCTs should allow narrowing the range of the actual risk, which would determine the need for changes, if any, in current transfusion practice. The effect of optimal RBCs storage may well vary according to different clinical settings.

If transfusion of old RBCs is shown to pose clinically significant risks, current systems of blood collection, storage and transfusion would need to be revised and would probably entail considerable operational and financial impacts. It would be premature to jeopardize the currently available blood supply based on retrospective clinical studies with marginal results or equivocal evidence (Dzik, 2008). Neither should fresher blood be withheld from small, well defined, particularly vulnerable patient groups based on negative RCTs that are not designed to discriminate among clinically relevant and important difference in outcome (Flegel, 2012). However, at this time blood collectors can address the storage lesion and clinicians can model systems and investigate strategies to use fresher blood in order to prepare for the possibility of a shortened RBC shelf life.

The decision regarding appropriate RBC storage time at transfusion currently relies on clinical judgment derived by combining the evidence from *in vitro*, *in vivo*, pre-clinical and prospective observational studies while several RCTs are in progress. A safe and practical approach might be to resort to the oldest RBCs, such as those within the last 7 days of the approved shelf life, only if there are national shortages. Resolution of the importance of the storage lesion may require large pragmatic clinical trials. In the meantime, it seems prudent to make plans and test models for using fresher RBCs without disrupting blood availability while the evidence for or against is being gathered.

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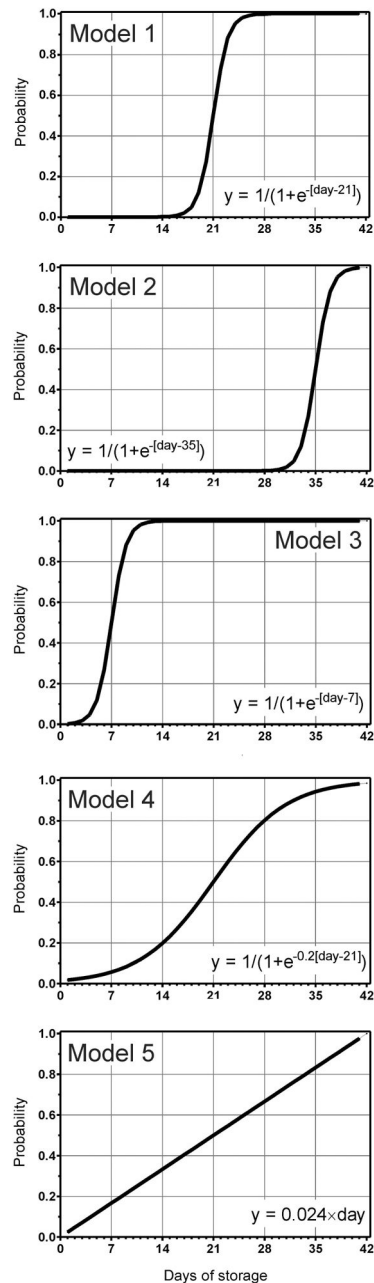


Fig. 1. Possible temporal patterns connecting red blood cell (RBC) storage time with outcome. The hypothetical models represent 5 different temporal patterns linking the RBC storage time with the probability of adverse clinical outcomes. Modified and reprinted from: Pereira, A. (2013) Will clinical studies elucidate the connection between the length of storage of transfused red blood cells and clinical outcomes? An analysis based on the simulation of randomized controlled trials. *Transfusion*, **53**, 34–40. with permission from Wiley.

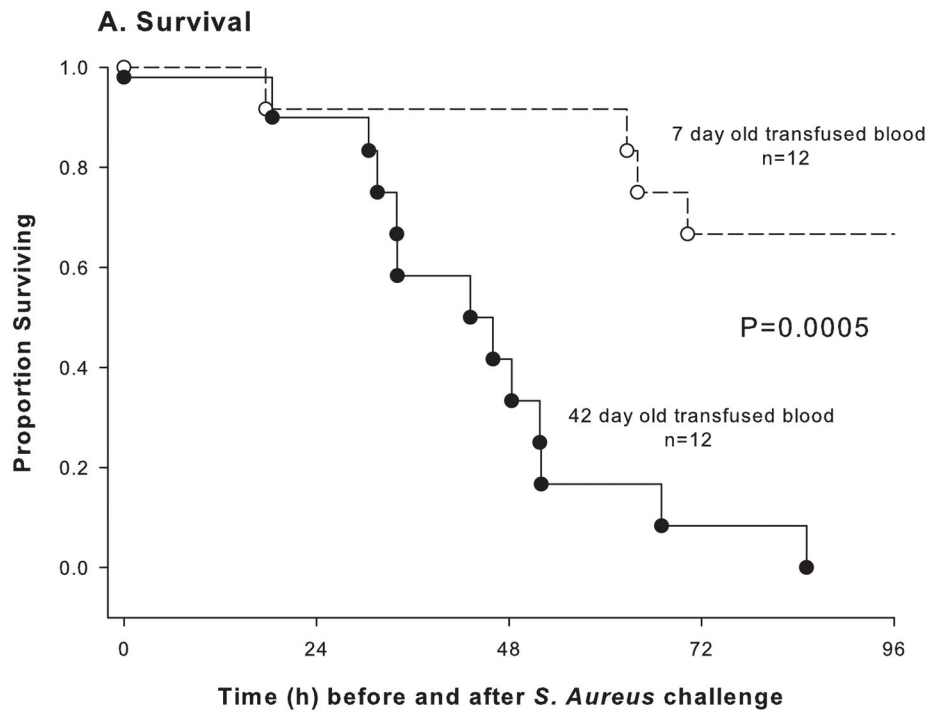


Fig. 2. Survival curves in *Staphylococcus aureus*-challenged dogs. Kaplan-Meier plots over the 96 h course of 12 dogs exchange transfused with 42-day-old red blood cells (RBCs; solid circle, solid line) and 12 dogs exchange transfused with 7-day-old RBC (open circle, dashed line). Reprinted from Solomon *et al.*, (2013).

Changes occurring in the RBC		Oxidative stress	
Echinocytes, reversible ↑	CD47 ↓	Protein oxidation ↑	
Sphero-echinocytes, irreversible ↑↑	Deformability ↓	Lipid peroxidation ↑	
Osmotic fragility ↑	Oxygen delivery ↓	Prostaglandin and isoprostanes ↑	
Microvesicles (procoagulant) ↑	Na-K-ATPase ↓		
Membrane rigidity ↑			
Phosphatidylserine exposure ↑			
Oxygen affinity of haemoglobin ↑			
Vascular endothelium adherence ↑			
Metabolic changes		Changes in the additive solution	
Lactate ↑	2,3-DPG ↓	K ⁺ ↑ and H ⁺ ↑	pH ↓
	Phosphate ↓	free haemoglobin (NO scavenger) ↑	complement ↓
	ATP, ADP, AMP ↓	free haem & iron (redox injury) ↑	opsonizing ability ↓
	Glutathione ↓	soluble lipids (platelet activating factor) ↑	lytic ability ↓
	S-nitrosohaemoglobin ↓	phospholipid vesicles ↑	
	Nitric oxide (NO) ↓	cytokines (IL1, IL6, IL8, TNF) ↑	
		histamines ↑ and enzymes ↑	
		complement split products ↑	

Fig. 3.

The RBC storage lesion. The changes affect the RBC cytoplasm and more so the membrane, parts of which are shed into the supernatant as microvesicles.

RBC, red blood cell; 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IL, interleukin; TNF, tumour necrosis factor.

Table I

Examples for RBC storage times currently approved and applied depending on the additive solution

<u>Additive solution* and shelf life (days)</u>		
Approved	Applied in clinical service	Country
49 days	49 days	Germany and Switzerland ¹
49 days	42 days	Germany ¹
42 days	42 days	Canada, China, Malaysia, and USA; ² Thailand; ^{2,3} Argentina, Australia, Austria, Brazil, France, Germany, India, Italy, Saudi-Arabia, Switzerland, Spain, and Tunisia ³
42 days	35 days	China; ^{2,3,4} Netherlands and United Kingdom (uniform for the countries); ³ Germany (large blood services for >20 years) ³
42 days	21 days	Japan ⁴
35 days	35 days	Brazil, China, Ethiopia, India, Malaysia, Oman, Pakistan, and Tunisia ⁵
21 days	21 days	China and Saudi-Arabia (few blood services) ⁶

*¹PAGGS-M (phosphate-adenine-glucose-guanosin-saline-mannitol, 285 mOsm/l isotonic): approved for a shelf life of 49 days; ²AS-3 (saline-adenine-glucose-citrate-phosphate): 42 days; ³SAGM (saline-adenine-glucose-mannitol, 376 mOsm/L hypertonic): 42 days; ⁴MAP (mannitol-adenine-phosphate): 42 days;;; ⁵CPDA-1 (citrate-phosphate-dextrose-adenine): 35 days; ⁶CPD (citrate-phosphate-dextrose): 21 days.

Table II

Completed randomized controlled trials without an effect of red blood cell storage time on outcome in different clinical settings

Reference	Patients		Clinical setting	Primary outcome parameters	Trial registration and acronym
	Treatment arm (n)	Control arm (n)			
Wasser <i>et al.</i> (1989)	118	119	Cardiac surgery	Postoperative bleeding	
Schulman <i>et al.</i> (2002)	8	9	Trauma	Mortality, infectious complications, respiratory failure	
Fernandes <i>et al.</i> (2001)	10	5	ICU with sepsis	Oxygen consumption, gastric mucosal pH change	
Walsh <i>et al.</i> (2004)	10	12	ICU with mechanical ventilation	gastric mucosal pH change, gastric to arterial PaCO ₂ gap	
Hebert <i>et al.</i> (2005)	26	31	ICU	Mortality, infections, thrombotic events, ischaemic stroke	
Fernandes da Cunha <i>et al.</i> (2005)	26	26	Premature infants <1.5 kg	Donor exposure, (not powered for) mortality	
2005–2007, unpublished	30	30	Traumatic brain injury	Cerebral oxygen extraction ratio	NCT00141674 *
Aubron <i>et al.</i> (2012)	25	26	ICU	Feasibility study, (not powered for) mortality	
Kor <i>et al.</i> (2012)	50	50	ICU with mechanical ventilation	Pulmonary function, (not powered for) mortality	TRALI2 trial NCT00751322
Heddle <i>et al.</i> (2012)	309	601	Acute care in-patients	Feasibility study, (not powered for) mortality	INFORM-P trial
Fergusson <i>et al.</i> (2012)	188	189	Premature infants <1.25 kg	Mortality, necrotizing enterocolitis, retinopathy, bronchopulmonary dysplasia, intraventricular haemorrhage	ARIP1 trial NCT00326924 ISRCTN65939658
Yürtük <i>et al.</i> (2013)	10	10	Haematology, out-patients	Sublingual microcirculation	

* recruiting completed in 2007, results not published. ICU, intensive care unit; PaCO₂, partial pressure of carbon dioxide in arterial blood.

Table III

Ongoing randomized controlled trials assessing the effect of red blood cell storage time on clinical outcomes

Official title or Acronym	Patients		Primary outcome	Country	Years	Trial registration
	Accrual goal (n)	Setting				
ABLE	2,510	ICU	all cause mortality at day 90	Canada 23 locations	2008–2013 completed*	ISRCTN44878718
Red Cell Storage Duration and Outcomes in Cardiac Surgery	2,800	All cardiopulmonary bypass patients	mortality at day 30 post-surgery	USA single centre	2007–2014 recruiting	NCT00458783
RECESS	1,696	Scheduled complex cardiac surgery	MODS	USA 26 locations	2010–2013 recruiting	NCT00991341
INFORM	24,400	Acute care inpatients	in-hospital mortality	Canada, USA, Australia	2012–2014 recruiting	ISRCTN08118744
TRANSFUSE	5,000	ICU excluding cardiac surgery	mortality at day 90	Australia, New Zealand, Finland	2012–2016 recruiting	NCT01638416 ACTRN12612000453886

* recruiting completed, publication of results pending. ICU, intensive care unit; MODS, multiple organ dysfunction score.

Table IV

Assessment of the effects of RBC storage time on various physiological parameters in healthy volunteers

Reference	Autologous RBC in crossover design		RBC characteristics		Leuco-reduction	Outcome parameters	Summary of results	Country	Trial registration
	Volun-teers (n)	RBC (n)	RBC storage time	RBC (n)					
Weiskopf <i>et al.</i> (2006)	9	2	3.4 h versus 23 days (median)	2	No	Anaemia-induced cognitive dysfunction	No difference in reversal of dysfunction	USA	
Hod <i>et al.</i> (2011)	14	2	3 to 7 days versus 40 to 42 days	2	Yes	Iron, extravascular haemolysis, metabolism parameters, inflammation, bacterial growth <i>in vitro</i>	Significant changes in iron and extravascular haemolysis parameters only	USA	NCT01319552
Berra <i>et al.</i> (2012)	9	1	3 days versus 40 days	1	Yes	Reactive hyperaemia index (reactive hyperperfusion)	No difference in hyperaemia index	USA	
Weiskopf <i>et al.</i> (2012)	35	2	1.7 h versus 24.5 days	2	Yes	Pulmonary function (gas exchange variables)	Equivalent slight declines in variables	USA	

RBC, red blood cell.