The effect of maximum storage on iron status, oxidative stress and antioxidant protection in paediatric packed cell units

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Background. Premature babies may receive multiple transfusions during the first weeks of their life. Strong associations exist between the receipt of blood transfusions and the development of the major consequences of prematurity such as retinopathy and chronic lung disease. The possible physiological link between the receipt of blood and disease is unclear, but iron-induced oxidative damage and/or bacterial colonisation would promote these conditions. Premature babies are poorly equipped to deal with any increases in iron and oxidative load that they may acquire via blood transfusions. To determine whether there are any relationships between these factors, we studied iron and oxidative status of just expired (i.e. 36 days old) paediatric red blood cell (RBC) packs.

Materials and methods. Just expired paediatric RBC packs were obtained from the local blood bank. The extracellular medium surrounding the RBC was separated by centrifugation and the following parameters measured: total iron concentration, total iron binding capacity, non-transferrin-bound iron [NTBI], haemoglobin, total and reduced ascorbate, and malondialdehyde concentration.

Results. The extracellular fluid of the paediatric packs (n =13) was rich in iron, a high percentage of which (36%) was present as potentially toxic NTBI. It was highly redox active with limited antioxidant protection and iron-binding capacity.

Discussion. The extracellular medium surrounding packed RBC could potentially be toxic if administered to patients with limited iron sequestering and antioxidant capacity, such as premature babies. Further studies are required to determine at what point during storage these changes become potentially harmful so that clinical studies can examine the optimal storage time for blood destined for premature babies.

Keywords: iron, oxidative stress, packed red blood cells, storage.

Introduction

Many premature babies are given multiple transfusions during the first few weeks of their life. Many studies have shown correlations between the receipt of blood transfusions and the development of the serious consequences of prematurity such as chronic lung disease (CLD), retinopathy (ROP), necrotising enterocolitis and intraventricular haemorrhage (IVH)[1-4]. The exact mechanism of this relationship is unclear, but a number of studies have suggested that transfusion-mediated iron overload and associated oxidative stress may be a major player in ROP[4,5], CLD[6], and IVH[7].

The clear benefits of restricting blood used in individual babies to that derived from a single donor[8] requires blood to be stored throughout the period that individual babies require transfusions. This adds the further complication of the influence of storage age of the blood used on clinical outcome[9,10]. The potential adverse effects of storage on the biochemistry and validity of stored erythrocytes has been given the term "the storage lesion"[11,12]. The involvement of iron and oxidative status in the storage lesion has received little attention, despite the potential adverse influence of the procedures involved in the preparation of packed cell units on the iron and oxidative status of the units.

The preparation of packed cell units involves the removal of most of the plasma proteins capable of binding and sequestering iron, such as transferrin and albumin, and also naturally occurring extracellular antioxidants, such as urate and ascorbate. Thus the potential for free unbound (potentially toxic) iron to build up in the additive solution and participate in poorly protected oxidative reactions clearly exists. This could have consequences on the viability of erythrocytes, on the behaviour of haemoglobin within the erythrocytes and on iron bioavailability[13-15]. In addition, the premature baby is poorly equipped to deal with any additional iron or oxidative load, being deficient in iron-binding

capacity[16-20] and antioxidant defences[1,20-22]. Consequently premature babies are likely to be at risk of transfusion-related iron and oxidative overload.

The purpose of this study was to determine the iron and oxidative status of paediatric packed red blood cell units stored for the maximum recommended time (35 days). The packs studied had, therefore, been stored for 36 days. The data from this study should provide the baseline information for subsequent studies investigating how any observed changes develop during storage for up to 35 days after donation and processing. This study also looked at storage for 42 days, the maximum recommended storage time for packed cell units used in adults, since adverse outcomes have been noted in seriously ill adults receiving older packed cell units[23,24].

To date few studies have investigated the iron and oxidative status of stored packed cell units. One study[25] showed increases in non-transferrin-bound iron (NTBI) during storage of packed cell units similar to those examined in this study. Iron-binding capacity was low and the saturation of residual transferrin high. Work on the involvement of oxidative damage demonstrated that membrane phospholipids in erythrocytes underwent oxidative modification with the release of malondialdehyde (MDA) during storage[26], and that the addition of iron chelators or some antioxidants reduced the degree of lipid peroxidation in stored erythrocytes[27-29]. To date, no study has examined the relationship between red blood cell viability, total iron levels, iron binding capacity, NTBI, oxidative stress and the levels of antioxidants in the supernatant of stored packed cell preparations destined for use in the neonatal intensive care unit. This study has done this by examining, in the extracellular fluid surrounding the erythrocytes, the following parameters: total iron concentration, iron binding capacity, haemoglobin (Hb) concentration (as a marker of the degree of erythrocyte membrane damage), NTBI concentration, total and reduced ascorbate concentration, and a marker of lipid peroxidation, MDA.

Materials and methods

Preparation of the extracellular phase for biochemical measurements

The packed cell preparations used in the neonatal units in our area are prepared from adult 450 mL whole blood units anticoagulated with citrate, phosphate and dextrose (CPD) and leucoreduced. The unit is hard spun to sediment the red blood cells and most of the plasma is removed to leave about 200 mL of red blood cells, to which 100 mL of saline, adenine, glucose, mannitol (SAGM) are added. The unit is then split to produce four to eight paediatric packs. The packs used in this study were collected from the Blood Bank at Derriford Hospital in Plymouth immediately after they had reached their expiry date (35 days). The blood analysed in this study had, therefore, been stored for 36 days. The packed cell units were gently inverted a few times to mix the contents. The blood was removed and centrifuged in plain vacutainers containing a serum separator. The tubes were centrifuged at 1,500 × *g* for 10 minutes to separate the packed cells from the extracellular phase[25], which was then removed. A sample of 400 μL of this extracellular phase was taken and added to 400 μL of 10% metaphosphoric acid (MPA) for measurement of ascorbic acid. This was centrifuged at 10,000 × *g* at 4 °C for 10 minutes and the supernatant stored at −80 °C for later analysis. The remainder of the extracellular phase from the original centrifugation was stored at −80 °C prior to the other biochemical analyses.

Total extracellular iron and total iron binding capacity

These two parameters were assessed by a scaled down version of the methods recommended by the International Committee for Standardisation in Haematology[30,31]. Four hundred microliters of extracellular phase, blanks and standards in SAGM were added to 400 μL of protein precipitation solution (0.6 M TCA and 0.4 M thioglycolic acid in 1 M HCl). This was mixed thoroughly for 1 minute and incubated at 56 °C for 15 minutes in a water bath. The samples were cooled and centrifuged for 5 minutes at 1,000 x *g* to provide an optically clear supernatant before adding 500 μL of this supernatant to 500 μL of ferene {0.5 mM 3-(2-pyridyl-5,6-bis-[2-5-furyl sulphonic acid]-1,2,4 triazine} in 1.5 M sodium acetate. This was incubated for 5 minutes before absorbance was measured at 593 nm in a spectrophotometer. Iron concentration was computed from the absorbance of standards included in each batch of samples.

To determine iron binding capacity, 350 μL of extracellular phase were added to 350 μL of iron saturating solution (100 μM FeCl in 5 mM HCl) and then mixed and allowed to stand at room temperature for 5 minutes. Light magnesium carbonate (35 mg) was added and the mixture agitated for 30 minutes. The magnesium carbonate was then removed by two sequential centrifugation steps of 1,000 × *g* for 5 minutes. Four hundred microliters of the resultant supernatant were removed for the measurement of iron as described above.

Non-transferrin-bound iron

NTBI was measured using a slight modification of the high performance liquid chromatography (HPLC) methods described by Kime *et al*.[32] and Paffetti *et al*[33]. Briefly, 400 μL of extracellular phase were incubated

with 40 µL of 0.8 M nitrilotriacetic acid (NTA) for 20 minutes at room temperature to chelate loosely bound iron, such as that chelated with residual citrate or albumin. The samples were then placed in 30 kDa Amicon Ultra 0.5 mL filters (Millipore, Nottingham, United Kingdom) and centrifuged at 13,000 × *g* at 4 °C for 30 minutes. Two hundred and fifty microliters of the ultrafiltrate were removed and incubated with 25 µL of 35 mM 3-hydroxy-1-propyl-2-methyl-pyridon-4-one for 5 minutes before injection into the HPLC system (sample loop 20 µL). The mobile phase consisted of 5 mM PIPES buffer pH7.0 containing 3.5 mM 3-hydroxy-1-propyl-2-methyl-pyridon-4-one and 5% acetonitrile. The 100 mm × 5 mm C18 column (Hichrom, Theale, Reading, United Kingdom) used was lined with polyetheretherketone (PEEK) and all tubing was PEEK. The mobile phase was pumped at a flow rate of 1 mL/minute using a Dionex pump (ThermoFisher, Loughborough, United Kingdom). The absorbance of the iron-chromophore complex was determined using a Dionex UV/VIS detector at a wavelength of 450 nm, and chromatography conducted using Chromeleon software (ThermoFisher). The concentration of NTBI was computed from blanks and standards taken through the whole procedure with each batch of samples.

Measurement of ascorbate

Total and oxidised ascorbate were measured by a combination of the methods described by Sato *et al.*[34] and Mitton and Trevithick[35]. The HPLC system consisted of a 150 mm x 5 mm Hichrom C18 column and a Milton Roy (Sunderland, United Kingdom) Constametric pump coupled to a BAS (Kenilwoth, United Kingdom) LCD40 electrochemical detector set at a voltage of +0.6 V. The mobile phase consisted of 0.1M sodium acetate containing 0.54 mM EDTA and 1.5 mM octylamine as an ion pair reagent at a pH of 5.0. This was pumped at 1.0 mL/minute. Two samples of 90 µL were removed from the initial MPA extract. To one of these, 10 µL of 5% MPA were added. A further 200 µL of 5% MPA were added and the sample mixed and injected into the HPLC system (20 µL sample loop). This provided the value for reduced ascorbate. To the other aliquot, 10 µL of TCEP were added and the mixture incubated at room temperature for 20 minutes to convert all the oxidised ascorbate to the reduced form[34]. Two hundred microliters of 5% MPA were then added, the sample mixed and injected into the HPLC system. This provided a measure of total ascorbate. The degree of ascorbate oxidation was computed by subtracting the value for reduced ascorbate from that of the total ascorbate.

Measurement of oxidative stress

Dumaswala *et al.*[26] clearly showed that membrane phospholipids in erythrocytes undergo oxidative modification with the release of MDA during storage. MDA is the most robust marker of oxidative damage to phospholipids and is the most abundant product of lipid peroxidation. Thus MDA is an excellent marker of oxidative stress in stored erythrocytes and was measured in this study by the method of Agarwal and Chase[36] which we have used previously[1,22].

Measurement of extracellular haemoglobin

The integrity of erythrocyte membranes during storage was assessed by measuring the concentration of Hb in the extracellular phase. A very minor modification of the standard cyanohaemoglobin method of Hb measurement[37] was used. One hundred microliters of extracellular phase were added to 2.0 mL of Drabkin's reagent (Sigma-Aldrich, Poole, United Kingdom). This was incubated for 15 minutes at room temperature and the absorbance at 540 nm measured in a spectrophotometer. The level of Hb was computed from standards and blanks made up in SAGM fluid.

Statistical analysis

Descriptive and correlational analyses were computed using Microsoft Excel 2010.

Results

Iron and iron binding capacity

The concentrations of total iron, NTBI, Hb and MDA in the extracellular phase and the iron-binding capacity are shown in Table I. A high proportion of the iron present in the extracellular phase was in the form of the potentially toxic NTBI. This is reflected by the low iron-binding capacity. The presence of MDA in the extracellular phase indicates that oxidative damage (lipid peroxidation) to the erythrocyte membranes was occurring. Combining the data from 36 and 42

Table I - Iron, Hb and MDA.
The concentrations of total iron, NTBI, total iron binding capacity, haemoglobin and MDA in the extracellular phase after storage for 36 and 42 days. Data are presented as the mean ± SD of 13 packs.

Storage age (Days)	Total iron (µM)	Total iron binding capacity (µM/mL)	NTBI (µM)	Haemoglobin (g/dL)	MDA (µM)
36	33.55±7.731	0.00986±0.00222	12.129±2.942	0.1799±0.0481	9.197±7.297
42	39.46±8.846	0.00956±0.00351	14.279±2.954	0.2108±0.0895	13.13±6.129

days storage showed a significant positive correlation between total iron and MDA (r =0.727; P<0.001) and total iron and Hb (r =0.519; P <0.05), and Hb and MDA (r =0.435; P <0.05). These findings indicate that the leakage of iron and Hb into the extracellular phase probably resulted from oxidative damage to the erythrocyte membranes during storage. Total Hb in the stored packs was remarkably consistent 27.99 g/dL±1.021 (SD) [n =9], reflecting the high haematocrit. The percentage loss of Hb into the extracellular fluid ranged from 0.38%-0.88% at 36 days storage and 0.39%-1.25% at 42 days.

Ascorbate and redox status

The significant inverse correlation between total ascorbate levels and MDA (r =−0.348; P <0.05) supported the suggestion that oxidative damage to the red blood cells contributed to the loss of Hb into the extracellular fluid. Further data on ascorbate status is shown in Table II; the small amount of ascorbate present was highly oxidised confirming poor antioxidant protection. The lower level of total ascorbate in samples stored for 42 days is slightly puzzling since the major effect of storage would be oxidation of ascorbate[38]. Consequently this should be fully reduced following incubation with TCEP. However, the oxidised form of ascorbate, dehydroascorbate, is itself susceptible to degradation during storage and can be rapidly and irreversibly hydrolysed to 2,3-diketogulonic acid at neutral pH, but not in acid conditions[39]. It is possible that hydrolysis of dehydroascorbate during storage between 36 and 42 days accounted for the loss of reducible ascorbate. Not only is ascorbate present in a highly oxidised state, but the addition of 10 μM reduced ascorbate to the extracellular fluid from a random selection of packs stored for 36 (n =5) or 42 days (n =5) led to the oxidation of 60.47% and 77.37%, respectively, following incubation for 5 minutes at room temperature.

Nature of non-transferrin-bound iron

With regard to NTBI, NTA has been used to chelate loosely bound iron in plasma and serum. It is known that at the concentration used in this study it does not chelate iron bound to transferrin[32,33]. It is unclear whether it can chelate iron from Hb. To clarify the distribution of NTBI in this study we compared NTBI measured when NTA was incubated with the extracellular phase prior to filtration and subsequent to filtration. The results of this part of the study are shown in Table III. On the assumption that the 30 kDa filters retain Hb, the NTBI measured when NTA was added subsequent to filtration should include chelation from small molecular weight compounds such as residual citrate, but not to protein. This was arbitrarily labelled as "free" NTBI and the difference between total NTBI and "free" NTBI as "loosely bound". The results showed a significant proportion of total NTBI appeared as the "free" category, and not apparently associated with larger molecular weight compounds, possibly Hb. Alternatively, the "free" pool may contain smaller protein fragments caused by oxidative damage to Hb or other proteins capable of binding iron and which may escape a 30 kDa filter. To investigate this, an experiment was conducted measuring NTBI in the extracellular fluid of 36-day old red cell packs (n =12) by the standard procedure (NTA added prior to filtration) using 30 kDa and 10 kDa filters. There was no difference in the measured NTBI whether the fluid was filtered through 30 kDa filters (12.208 μM±2.425) or 10 kDa filters (12.886±2.251) (mean±SD). At present the nature of the "free" pool of NTBI is unclear. What is, however, clear is that a large proportion of iron in the extracellular phase following storage for 36 or 42 days is present as potentially toxic NTBI.

Table II - Total, reduced and oxidised ascorbate.
Concentration of total, oxidised and reduced ascorbate in the extracellular phase. Data are presented as the mean±SD (n =13 packs).

Storage age (days)	Total ascorbate (μM)	Reduced ascorbate (μM)	Oxidised ascorbate (μM)	Percentage oxidised
36	4.419±2.090	0.821±0.754	3.602±1.647	83.88±11.706
42	2.071±1.208	0.117±0.194	1.965±1.006	94.967±6.412

Table III - Distribution of NTBI.
Distribution of NTBI according to the presence or absence of NTA prior to filtration. Total NTBI is that measured when NTA is added prior to filtration. "Free" NTBI is that measured when NTA is added following filtration. "Loosely bound" is the difference between total and "free". Data are presented as the mean±SD (n=13). The percentage of total iron in the various pools is given in parentheses.

Storage age (days)	Total NTBI (μM)	"Free" NTBI (μM)	"Loosely bound" NTBI (μM)
36	12.129±2.942 (36.15%)	7.285±2.533 (21.71%)	4.84±1.518 (14.44%)
42	14.279±2.954 (36.18%)	9.912±2.198 (25.12%)	3.925±1.574 (9.95%)

Discussion

The results of this study indicate that the extracellular medium surrounding red blood cells in just expired paediatric packed cell preparations is rich in iron, highly redox active and provides limited antioxidant protection. The most likely explanation of these findings is that the lack of antioxidant protection has allowed oxidative damage to the erythrocyte membrane[13,15,27,28] thus enable release of Hb into the extracellular medium. A variety of oxidising agents are capable of causing the release of iron from erythrocytes[13,14]. Furthermore, the release of iron appears to be associated with the formation of methaemoglobin, which is a relatively unstable Hb derivative that readily frees the haem moiety from the haem pocket[11,15]. The iron that is released from Hb under these conditions appears to be redox active[13], and thus potentially damaging. There is evidence that free radical damage to stored erythrocytes may be mediated by iron released from oxidised haemoglobin[27]. Accordingly, studies have shown positive effects of some antioxidants and iron chelators in defending against lipid peroxidation in stored erythrocytes[27,29] and fresh erythrocytes challenged with free radicals *in vitro*[40]. Ascorbate is particularly adept at defending against oxidative damage to the Hb molecule[41]. Ascorbate is able to reduce the products of Hb oxidation and protect against their consequences, such as the release of redox active iron[41]. The ability of ascorbate to reduce methaemoglobin[42] and ferryl haemoglobin[41], another derivative of Hb oxidation[15], is particularly important. The relative paucity of ascorbate and the high degree of ascorbate oxidation would limit the protective action of ascorbate in packed cell preparations. It is, therefore, highly likely that oxidative damage to erythrocytes during storage[15] could give rise to the release of redox active iron within the erythrocyte which could further exacerbate oxidative damage leading to lipid peroxidation in the erythrocyte membrane, haemolysis and to some extent, the extracellular release of iron, establishing a potentially vicious cycle of events.

In this study we used extracellular Hb as a marker of haemolysis. Comparing the measured total Hb and extracellular Hb indicated that the apparent haemolysis in some of the packs analysed exceeded the permitted maximum of 0.8%. However, storage of packed cell units leads to the release of Hb-rich microparticles into the extracellular fluid[43]. Whether the Hb contained within microparticles can be considered as a valid marker of haemolysis is unclear, as the mechanism by which microparticles are formed is currently not fully established. Consequently, the calculated degree of haemolysis based on extracellular Hb as measured in this study may not be accurate. The relative amounts of free Hb and Hb contained within microparticles in this study are unknown. Previous studies found values ranging from 10%[43] to 50%[44] of Hb contained within microparticles. It is similarly unclear how iron is distributed between these two compartments. In this study, the level of extracellular Hb in comparison to the total iron level suggests that little iron remains bound to Hb. This is supported by the high level of NTBI we found. This would also imply that some iron leaves the Hb prior to the entry of Hb into the extracellular phase, possibly by the mechanisms discussed above. Nevertheless, the extracellular fluid contains a considerable amount of iron, and particularly NTBI, when compared to that normally found in plasma.

In this study we examined just expired paediatric packs. The standard practice in Neonatal Intensive Care Units is to use blood from a single donor for individual babies, meaning that some babies, particularly those receiving multiple transfusions, may be given blood with similar characteristics to those in this study. There is also a tendency to use older blood first. This could be potentially harmful in recipients who are poorly equipped to deal with additional iron and oxidative load such as premature babies. The first major study that pointed out that premature babies were poorly equipped to deal with such events was that by Sullivan[20]. Since then a number of studies have reinforced this original observation. The most recent study[45] showed that the values of iron and percentage iron-binding in premature babies were higher than the normal reference values. This increase was particularly marked in male babies, who tend to show a greater degree of morbidity than their female counterparts[46,47]. In addition to specific iron binding to transferrin, albumin may also play a role as an antioxidant by binding free iron and limiting the ability of iron to generate free radicals[48]. The ability of albumin to bind iron seems to be particularly important as a defence mechanism against iron-induced oxidative damage[49]. Studies have shown significantly lower serum albumin levels in premature babies than in babies born at term[19]. Serum albumin in premature babies is particularly susceptible to oxidative damage[50] which would further limit its ability to bind iron. The level of caeruloplasmin, which converts iron to the form necessary to bind to transferrin, may also be low in premature babies[18]. It, therefore, appears that the processes that operate to sequester free iron, such as transferrin, caeruloplasmin and albumin, are limited in the premature baby. Premature babies also have limited antioxidant defences to protect against circulating free radicals[1,20,51-55]. Furthermore, the concentration of the low molecular weight antioxidants ascorbate, urate and, possibly, glutathione in serum and bronchoalveolar lavage fluid in premature babies falls during the first week of life, recovering over the next few weeks[1,21,51,53,55].

Premature babies who require blood transfusions will receive their first transfusion, and possibly the majority of their treatments, within the first week of life. Thus the receipt of blood, with the possibility of generating excessive free radicals, coincides with a period when antioxidant protection is falling. Consequently, the premature baby will be particularly susceptible to adverse reactions to transfused iron because of their poor antioxidant status and inability to deal with any excess iron.

In addition to the above, extracellular Hb is also potentially toxic to a number of tissues including those of the cardiovascular and respiratory systems[56,57].

The mechanisms by which extracellular Hb is capable of causing tissue damage are complex, varied and incompletely understood. However, the addition of around 25 mg of Hb per Kg body weight through transfusion (based on data from this study), and the addition of further Hb through post-transfusion haemolysis of the blood received[58] provides a further route through which transfusion-related tissue damage may occur.

What is now required is a study of these parameters in stored paediatric packs from receipt at the blood bank (usually 3 days after donation) up until 35 days. Such a study should identify a maximum storage age at which these changes are minimal. This should then be followed by a prospective study into the clinical outcome in babies receiving blood stored for the minimum time compared with babies receiving blood transfusions according to current practice. An alternative approach could be to re-examine the preparation of the packed cell units to attempt to protect against adverse effects of storage mediated by iron and free radicals. The importance of iron in neonatal development[59] would preclude the use of iron chelators. However, the addition of appropriate antioxidant protection and/or apotransferrin might be worth examining. Monitoring the iron status and iron-binding capacity of currently available packed cells prior to use would also be desirable, but may be difficult to achieve in the clinical setting. The overall aim is to try to improve clinical outcome in this very vulnerable group of patients. The findings of this study might also be relevant to adverse events following packed cell transfusion in critically ill adults[23,24,60,61], since units used in adult transfusion may be stored for up to 42 days.

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References

1) Collard KJ, Godeck S, Holley JE, Quinn MW. Pulmonary antioxidant levels and oxidative damage in ventilated premature babies. Arch Dis Child 2004; **89**: F412-6.
2) Baer VL, Lambert DK, Henry E, et al. Among very-low-birth-weight neonates is red blood cell transfusion an independent risk factor for subsequently developing a severe intraventricular hemorrhage? Transfusion 2011; **51**: 1170-8.
3) El-Dib M, Narang S, Lee E, et al. Red blood cell transfusion, feeding and necrotizing enterocolitis in preterm infants. J Perinatol 2011; **31**: 183-7.
4) Romagnoli C. Risk factors and growth factors in ROP. Early Hum Devel 2009; **85**: S79-82.
5) Akkoyun I, Oto S, Yilmaz G, et al. Risk factors in the development of mild and severe retinopathy of prematurity. J Am Ass Pediatr Ophthalmol Strabismus 2006; **10**: 449-53.
6) Collard KJ. Is there a causal relationship between the receipt of blood transfusions and the development of chronic lung disease of prematurity? Med Hypoth 2006; **66**: 355-64.
7) Perrone S, Tataranno ML, Negro S, et al. Early identification of the risk of free radical-related diseases in preterm newborns. Early Hum Devel 2010; **86**: 241-4.
8) Bell EF. When to transfuse preterm babies. Arch Dis Child Fetal Neonatal Ed 2008; **93**: F469-73
9) Gauvin F, Spinella PC, Lacroix J, et al. Association between length of storage of transfused red blood cells and multiple organ dysfunction syndrome in pediatric intensive care patients. Transfusion 2010; **50**: 1902-13.
10) Karam O, Tucci M, Bateman ST, et al. Association between length of storage of red blood cell units and outcome of critically ill children: a prospective observational study. Crit Care 2010; **14**: R57-64.
11) Hess JR. Red cell changes during storage. Transfus Apher Sci 2010; **43**: 51-9.
12) Triulzi DJ, Yazer MH. Clinical studies of the effect of blood storage on patient outcomes. Transfus Apher Sci 2010; **43**: 95-106.
13) Ferrali M, Signorini C, Ciccoli L, Comporti M. Iron release and membrane damage in erythrocytes exposed to oxidising agents, phenylhydrazine, divicine, and isouramil. Biochem J 1992; **285**: 295-301.
14) Comporti M, Signorini C, Buonocore G, Ciccoli L. Iron release, oxidative stress and erythrocyte ageing. Free Rad Biol Med. 2002; **32**: 568-76.
15) Kanias T, Acker JP. Biopreservation of red blood cells - the struggle with haemoglobin oxidation. FEBS J 2010; **277**: 343-56.
16) Moison RMW, Hasnoot AA, Van Zoren-Grobben D, Berger HM. Plasma proteins in acute and chronic lung disease of the newborn. Free Rad Biol Med 1998; **25**: 321-8.
17) Lackmann GM, Hess L, Tollner U. Reduced iron-associated antioxidants in premature newborns suffering intracerebral haemorrhage. Free Rad Biol Med 1996; **20**: 407-9.
18) Lindeman JHN, Lentjes EGWM, Van Zoeren-Grobben D, Berger HM. Postnatal changes in plasma caeruloplasmin and transferrin antioxidant activities in preterm babies. Biol Neonate 2000; **78**: 73-6.
19) Galinier A, Periquet B, Lambert W, et al. Reference range for micronutrients and nutritional marker proteins in cord blood of neonates appropriated for gestational ages. Early Hum Devel 2005; **81**: 583-93.
20) Sullivan JL. Iron, plasma antioxidants, and the 'oxygen radical disease of prematurity. Am J Dis Child 1988; **142**: 1341-4.
21) Vyas JR, Currie A, Dunster C, et al. Ascorbic acid concentration in airways lining fluid from infants who develop chronic lung disease of prematurity. Eur J Pediatr 2001; **160**: 177-84.
22) Collard KJ, Godeck S, Holley JE. Blood transfusion and

pulmonary lipid peroxidation in ventilated premature babies. Pediatr Pulmonol 2005; **39**: 257-61.
23) Koch CG, Sessler DI, Figueroa P, et al. Duration of red-cell storage and complications after cardiac surgery. N Eng J Med 2008; **358**: 1229-39.
24) Tinmouth A, Fergusson D, Chin Yee I, Hebert PC. Clinical consequences of red cell storage in the critically ill. Transfusion 2006; **46**: 2014-27.
25) Marwah SS, Blann A, Harrison P, et al. Increased non-transferrin bound iron in plasma depleted SAG-M red blood cell units. Vox Sang 2002; **82**: 122-6.
26) Dumaswala UJ, Zhuo L, Jacobsen DW, et al. Protein and lipid oxidation of banked human erythrocytes: role of glutathione. Free Rad Biol Med 1999; **27**: 1041-9.
27) Knight J, Voorhees RP, Martin L. The effect of metal chelators on lipid peroxidation in stored erythrocytes. Ann Clin Lab Sci 1992; **22**: 207-13.
28) Knight J, Searles DA, Clayton FC. The effect of desferrioxamine on stored erythrocytes: lipid peroxidation, deformity and morphology. Ann Clin Lab Sci 1996; **26**: 283-90.
29) Knight JA, Searles DA. The effects of various antioxidants on lipid peroxidation in stored whole blood. Ann Clin Lab Sci 1994: **24**: 294-301.
30) International Committee for Standardisation in Haematology. The measurement of total and unsaturated iron-binding capacity in serum. Br J Haematol 1978; **38**: 281-90.
31) Iron panel of the International Committee for Standardisation in Haematology. Revised recommendations for the measurements of serum iron in human blood. Br J Haematol 1990; **75**: 615-6.
32) Kime R, Gibson A, Yong W, et al. Chromatographic method for the determination of non-transferrin-bound iron suitable for use on the plasma and bronchoalveolar lavage fluid of preterm babies. Clin Sci 1996; **91**: 633-9.
33) Paffetti P, Perrone S, Longini M, et al. Non-protein bound iron detection in small samples of biologic fluids and tissues. Biol Trace Elem Res 2006; **112**: 221-32.
34) Sato Y, Uchiki T, Iwama M, et al. Determination of dehydroascorbic acid in mouse tissues and plasma by using tris(2-carboxyethyl) phosphine hydrochloride as reductasnt in metaphosphoric acid/ ethylenediaminetetraacetic acid solution. Biol Pharm Bull 2010; **33**: 364-9.
35) Mitton K, Trevithick J. High performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, tocopherol and ascorbate. Methods Enzymol 1994; **233**: 523-39.
36) Agarwal R, Chase S. Rapid, fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples. J Chromatogr B Analyt Technol Biomed Life 2002; **775**: 121-6.
37) Moore GL, Ledford ME, Merydith A. A micromodification of the Drabkin haemoglobin assay for measuring plasma haemoglobin in the range of 5 to 2000 mg/dl. Biochem Med 1981; **26**: 167-73.
38) Karlsen A, Blomhoff R, Gundersen TE. Stability of whole blood and plasma ascorbic acid. Eur J Clin Nutr 2007; **61**: 1233-6.
39) Lykkesfeldt J, Loft S, Poulsen HE. Determination of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography with coulometric detection - are they reliable biomarkers of oxidative stress? Anal Biochem 1995; **229**: 329-35.
40) Ertabak A, Kutluay T, Unlu A, et al. The effect of deferrioxamine on peroxynitrite-induced oxidative damage in erythrocytes. Cell Biochem Funct 2004; **22**: 149-52.
41) Dunne J, Caron A, Menu P, et al. Ascorbate removes key precursors to oxidative damage by cell free haemoglobin *in vitro* and *in vivo*. Biochem J 2006; **399**: 513-24.
42) Donadee C, Raat NJH, Kanias T et al. Nitric oxide scavenging by red blood cell microparticles and cell-free haemoglobin as a mechanism for the red cell storage lesion. Circulation 2011; 124: 465-476.
43) Greenwalt TJ, McGuiness CG, Dumasawala UJ. Studies in red blood cell preservation, 4: plasma vesicle haemoglobin exceeds free haemoglobin. Vox Sang 1991; **61**: 14-7.
44) Dunne J, Svistunenko DA, Alayash AI, et al. Reactions of cross-linked methaemoglobin with hydrogen peroxide. Adv Exp Med Biol 1999; **471**: 9-15.
45) Molloy EJ, El-Khuffash A, Bieda A, et al. Elevated iron indices in preterm infants: association with male gender. Am J Perinatol 2009; **26**: 7-10.
46) Zisk JL, Genen LH, Kirkby S, et al. Do premature female infants really do better than their male counterparts? Am J Perinatol 2011; **28**: 241-6.
47) Binet ME, Bujold E, Lefebvre F, et al. Role of gender in morbidity and mortality of extremely premature neonates. Am J Perinatol 2012; **29**: 159-66.
48) Fukuzawa K, Saitoh Y, Akai K, et al. Antioxidant effect of serum albumin on membrane lipid peroxidation by iron chelates and superoxide. Biochim Biophys Acta 2005; **1668**: 145-55.
49) Loban A, Kime R, Powers H. Iron binding antioxidant potential of plasma albumin. Clin Sci (Lond) 1997; **93**: 445-51.
50) Marzocchi B, Perrone S, Paffetti P, et al. Nonprotein-bound iron and plasma protein oxidative stress at birth. Pediatr Res 2005; **58**: 1295-9.
51) Silvers KM, Gibson AT, Russell JM, Powers HJ. Antioxidant activity, packed cell transfusions, and outcome in premature infants. Arch Dis Child 1998; **78**: F214-9.
52) Turgut M, Basaran O, Cekman M, et al. Oxidant and antioxidant levels in preterm newborns with idiopathic hyperbilirubinaemia. J Paediatr Child Health 2004; **40**: 633-7.
53) Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. Semin Fetal Neonatal Med 2010; **15**: 191-5.
54) Nassi N, Ponziani V, Becatti M, et al. Anti-oxidant enzymes and related elements in term and preterm newborns. Pediatr Int 2009; **51**: 183-7.
55) Rook D, Te Braake FWJ, Schierbeek H, et al. Glutathione synthesis rates in early postnatal life. Pediatr Res 2010; **67**: 407-11.
56) Reeder BJ. The redox acivity of hemoglobins: From physiologic functions to pathologic mechanisms. Antioxid Redox Signal 2010; **13**: 1087-23.
57) Buehler PW, D'Agnillo F. Toxicological consequences of extracellular haemoglobin: biochemical and physiological perspectives. Antioxid Redox Signal 2010; **12**: 275-91.
58) Hod EA, Brittenham GM, Billote GB, et al. Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron. Blood 2011; **118**: 6675-82.
59) Collard KJ. Iron homeostasis in the neonate. Pediatrics 2009; **123**: 1208-16.
60) Scott BH, Seifert FC, Grimson R. Blood transfusion is associated with increased resource utilisation, morbidity and mortality in cardiac surgery. Ann Card Anaesth 2008; **11**: 15-9.
61) Sanders J, Patel S, Cooper J, et al. Red blood cell storage is associated with length of stay and renal complications after cardiac surgery. Transfusion 2011; **51**: 2286-94.

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