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

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Background. Receipt of blood transfusions is associated with the major consequences of prematurity such as bronchopulmonary dysplasia. Transfusion-mediated (iron-induced) oxidative damage, coupled with the limited ability of the premature baby to deal with enhanced iron and oxidative load may contribute to this. Adverse effects of transfusion may be related to duration of storage. This study examined the influence of storage on iron and oxidative status in paediatric packed red blood cell units.

Materials and methods. Paediatric packed red blood cell units were sampled 3 days post-donation, then at 7 days and weekly until day 35. The extracellular medium was separated and the following measured: total iron concentration, total iron binding capacity, non-transferrin-bound iron, haemoglobin, total and reduced ascorbate, glutathione and malondialdehyde.

Results. Measurable total and non-transferrin bound iron were present in the extracellular fluid of paediatric packs on day 3. Both parameters rose almost linearly to maximal values at 35 days. Haemoglobin and malondialdehyde levels rose gradually from day 3 to day 21, then more steeply to day 35. Ascorbate existed mainly in the oxidised form and fell rapidly towards the end of storage. Intracellular GSH fell throughout the period of storage. Strong correlations existed between biomarkers of oxidative damage and iron parameters.

Discussion. These data suggest that iron released following the initial preparation of packed red blood cell units may derive from free radical-mediated oxidative damage to the red blood cells and haemoglobin, rather than from extracellular haemoglobin. Iron continues to be released during storage as antioxidant protection declines. A cycle of free radical-mediated damage may initiate and then further exacerbate iron release during storage which, in turn, may mediate further free radical-mediated cellular damage. The potential consequences to recipients of older stored blood may be significant.

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

Introduction

Many premature babies are given multiple transfusions during their first few weeks of life. A number of 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 major players 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 the naturally occurring extracellular anti-oxidants 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 can have consequences on the viability of erythrocytes, and 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 capabilities[16-20] and anti-oxidant defences[1,20-22]. Consequently the premature baby is likely to be at risk of transfusion-related iron and oxidative overload.

A preliminary study in our laboratory confirmed that the extracellular fluid of immediately time-expired (36 days storage) paediatric packed cell units was rich in iron, a high percentage of which was in the form of the potentially toxic non-transferrin bound iron (NTBI)[23]. In addition, the fluid was highly redox active with limited anti-oxidant protection and iron-binding capacity[23]. The purpose of the current study was to further develop these findings by examining the changes in iron and oxidative status of paediatric packed cell units stored from the point of arrival at the Blood Bank (3 days after donation) up to the maximum recommended time (35 days). The study also builds on previous findings from related studies in other laboratories. For example, increases in NTBI in the extracellular medium during storage of packed cell units have been reported, along with the finding that iron-binding capacity was very low and the saturation of residual transferrin was very high[24]. Other studies demonstrated that membrane phospholipids in erythrocytes underwent oxidative modification with the release of malondialdehyde (MDA) during storage[25], and that the addition of iron chelators or some anti-oxidants reduced the degree of lipid peroxidation in stored erythrocytes[26-28]. To date, no study has examined the relationship between erythrocyte viability, total iron levels, iron-binding capacity, NTBI, oxidative stress and the levels of anti-oxidants in the supernatant of stored paediatric packed cell preparations from arrival at the Blood Bank until time expiry (35 days). This study has done this by examining, in the supernatant, 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 concentrations, total red blood cell and extracellular glutathione, and a marker of lipid peroxidation, MDA. The measurement of these related parameters in the same packs has allowed a reassessment of the potential mechanism by which adverse effects may occur during storage and how they may affect the recipients.

Materials and methods

Preparation and storage of packed cell units

Paediatric packed cell units were prepared at the NHS Blood and Transplant Centre (Bristol, UK) as described previously[23]. The adult blood is anticoagulated with citrate, phosphate, dextrose (sodium citrate 89 mmol/L; citric acid 16 mmol/L; glucose 128 mmol/L; sodium phosphate 16 mmol/L) at a ratio of 63 mL anticoagulant to 450 mL blood. The blood is filtered to remove leucocytes and centrifuged to yield the red blood cells. Most of the plasma is removed (leaving about 20 mL) and replaced with 100 mL of SAGM additive (NaCl 150 mmol/L; adenine 1.25 mmol/L; glucose 45.4 mmol/L; mannitol 28.8 mmol/L). This provides a unit containing approximately 200 mL of red blood cells and 100 mL of additive. The adult pack is then split into six paediatric units of 45-50 mL. In this study ten adult packs were used providing ten sets of paediatric packs for study. Each batch of six paediatric units was transported using the regular NHS blood transport carriers to the blood centre at Derriford Hospital (Plymouth, UK), and stored in the hospital blood bank in a quarantined section in the same refrigerator that houses units for clinical use. One pack was removed from each set on arrival at the blood bank (3 days after donation), one at 7 days after donation, and then one every 7 days up to 35 days storage.

Preparation of extracellular phase for biochemical measurements.

The packed cell units were gently inverted a few times to mix the contents. The blood was removed and centrifuged in plain vacutainers. The tubes were centrifuged at 1,500×*g* for 10 minutes to separate the packed cells from the extracellular phase[24]. The extracellular phase was removed, 400 µL of this were removed and added to 400 µL of 10% metaphosphoric acid (MPA) containing 2 mM ethylene diamine tetra-acetic acid (EDTA) A for measurement of ascorbic acid. The MPA extract 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 as described previously[23]. Briefly, 400 µL of extracellular phase, blanks and standards in SAGM were added to 400 µL of protein precipitation solution (0.6M trichloroacetic acid and 0.4M thioglycolic acid in 1M 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×*g* to provide an optically clear supernatant. Five hundred microlitres of this were added to 500 µL of ferene {0.5 mM 3-(2-pyridyl-5,6-bis-[2-5-furyl sulphonic acid]-1,2,4 triazine} in 1.5M sodium acetate. This was incubated for 5 minutes before absorbance was measured at 593 nm in a spectrophotometer. The 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). This was mixed and allowed to stand at room temperature for 5 minutes. Next, 35 mg of light magnesium carbonate 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. Finally, 400 µL 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 of Kime *et al*.[29] and Pafetti *et al.*[30] as described in detail previously[23]. Briefly, 400 µL of extracellular phase were incubated with 40 µL of 0.8M 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, Watford, UK) and centrifuged at 13,000×*g* at 4 °C for 30 minutes. Two hundred and fifty microlitres 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 being injected into the HPLC system (sample loop 20 µL). The mobile phase consisted of 5 mM PIPES buffer pH 7.0 containing 3.5 mM 3-hydroxy-1-propyl-2-methyl-pyridon-4-one and 5% acetonitrile. The column was a PEEK lined 100 mm × 5 mm C18 column (Hichrom). All tubing was PEEK. The mobile phase was pumped at a flow rate of 1 mL/minute using a Dionex pump. The absorbance of the iron-chromophore complex was determined using a Dionex UV/VIS detector at a wavelength of 450 nm and the chromatography conducted using Chromeleon software (Thermo Fisher Scientific, Loughborough, UK). 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 the method described by Sato *et al*[31]. Two aliquots 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, 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 tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (350 mM) were added and the mixture incubated at room temperature for 20 minutes to convert all the oxidised ascorbate to the reduced form[31]. Two hundred microlitres 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.

The HPLC system consisted of a 150 mm × 4.6 mm ACE 5 AQ C18 column (Hichrom, Reading, UK), a Milton Roy Constametric pump (Milton Roy, Sunderland, UK) coupled to a BAS LCD40 electrochemical detector (BASi, West Lafayette IN, USA) set at a voltage of +0.6 V. The mobile phase consisted of 50 mM phosphate buffer containing 0.54 mM EDTA and 2% methanol at pH 2.8. This was pumped at a rate of 1.0 mL/minute.

Measurement of glutathione

Glutathione was measured by a procedure based on those described by Kand'ar *et al.*[32] and Michaelsen *et al*[33]. For extracellular glutathione (GSH), 100 µL of sample, or standard or blank were added to either 100 µL of H_2O or 100 µL of 100 mM n-ethylmaleimide [NEM] in H_2O. The tubes were incubated at room temperature for 30 minutes, after which time 100 µL of trichloroacetic acid (0.1 g/mL in 2 mM EDTA) were added to each tube. The tubes were mixed well and centrifuged at 15,000×*g* for 5 minutes at 4 °C. Fifty microliters of supernatant were removed and added to 200 µL of o-phthaldialdehyde (OPA) derivatising solution (75 mM OPA in 0.125M borate buffer pH 9.5 containing 10% vol ethanol and 2 mM EDTA[33]). Samples were incubated for 2.5 minutes at room temperature before injection into the HPLC system. For the measurement of GSH in whole blood, 200 µL of thawed stored whole blood (−80 °C) were added to 200 µL of H_2O. The mixture was placed in 30 kDa Amicon Ultra filters (0.5 mL) (Millipore) and centrifuged at 13,000×*g* for 30 minutes to separate the particulate matter from the ultrafiltrate. Two 100 µL aliquots of ultrafiltrate were removed. One was added to 100 µL of H_2O, while the other was added to 100 µL of 100 mM NEM. The samples were incubated for 30 minutes at room temperature, after which time 100 µL of 0.1 g/mL trichloroacetic acid in 2 mM EDTA were added. The tubes were centrifuged at 15,000×*g* for 10 minutes at 4 °C. One hundred microlitres of supernatant were removed and diluted 1:10 with H_2O containing 2 mM EDTA. One hundred microlitres were removed and processed as described above.

The sample was analysed on a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) comprising an ultimate 3000 pump and fluorescence detector. Chromatography was controlled using Chromeleon software (version 6.8) (Thermo Fisher Scientific). Chromatography was conducted using a 100 mm × 4.6 mm Hichrom C18 column, protected by

a guard column of the same resin and a 2 µm inline filter (Hichrom, Theale, UK). The mobile phase was pumped at a rate of 1.0 mL/min. Glutathione was detected using an excitation wavelength of 338 nm and an emission wavelength of 458 nm.

Measurement of oxidative stress

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[34], which we have used previously[1,22]. In a 2.0 mL screw top centrifuge tube, 50 µL of plasma, standard or blank were added to 50 µL of butylated hydroxytoluene (BHT) (0.05% in 95% ethanol), followed by 400 µL of 0.44M phosphoric acid and 100 µL of 42.0 mM thiobarbituric acid. The tubes were capped, mixed well and heated for 60 minutes at 100 °C in a dry block. After cooling, 300 µL of n-1-butanol were added to the tubes and the tubes mixed well to permit the extraction of MDA into the butanol. The tubes were centrifuged at 13,000×*g* at 4 °C to separate the aqueous and butanol phases. Portions (20 µL) of the butanol extract were injected directly into the HPLC system, which was a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) comprising an ultimate 3000 pump and fluorescence detector. Chromatography was conducted using a 100 mm × 4.6 mm Hichrom C18 column, protected by a guard column of the same resin and a 2 µm inline filter (Hichrom). Chromatography was controlled using Chromeleon software (version 6.8) (Thermo Fisher Scientific). The mobile phase (50 mM potassium phosphate/methanol 80;20 v/v, pH 6.8) was pumped at a rate of 1.0 mL/min. MDA was detected using an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

Measurement of extracellular haemoglobin

The viability of the erythrocyte membranes during storage was assessed by measuring the level of Hb in the extracellular phase[35]. A very small modification was made to the standard cyanohaemoglobin method of Hb measurement[36]. A 100 µL portion of extracellular phase was added to 2.0 mL of Drabkin's reagent. 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.

Statistics

Descriptive and correlational analysis was completed using Stat 200 (Biosoft, Cambridge, UK).

Results

Total iron, non-transferrin bound iron and iron-binding capacity

Changes in the concentration of total iron and NTBI during storage are shown in Figure 1. Even on arrival at the Blood Bank (3 days after donation) there was already measurable iron and NTBI in the extracellular fluid surrounding the packed cells. From that point on the level of total iron rose almost linearly to a level of 32.75 µmol/L at maximal storage age. NTBI levels also rose throughout the storage period reaching a maximum level of 14.88 µmol/L on day 35. Thus 45.4% of the total iron in the extracellular medium on day 35 was in the potentially highly toxic NTBI form. From day 14 onwards NTBI represented 45-50% of the total iron in the extracellular fluid. It is no surprise therefore, that the high level of NTBI was associated with a predicted very low iron-binding capacity throughout the storage period (6.014±1.813 - 6.857±2.006 nmol/mL] and that it changed very little throughout the storage period.

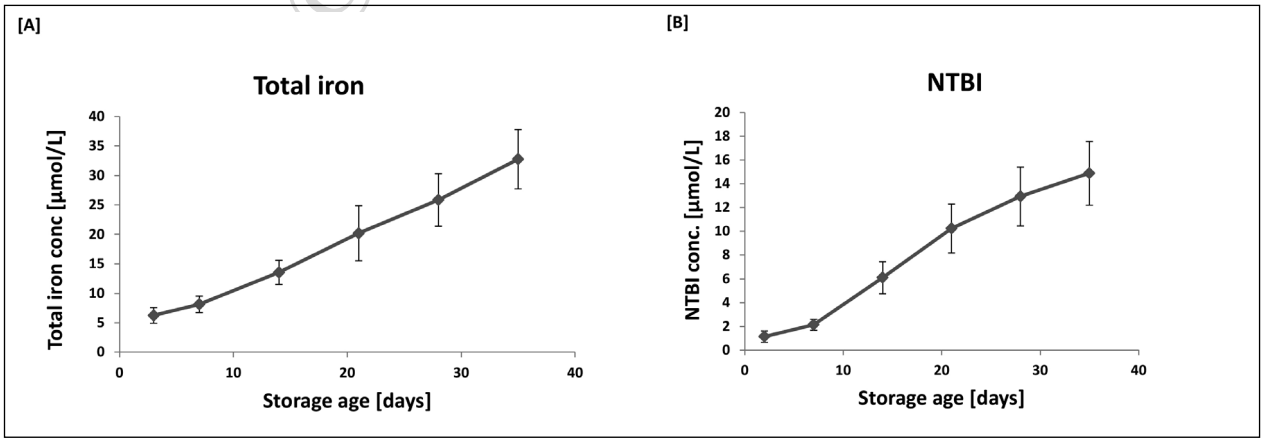

Figure 1 - Levels of total iron [A] and NTBI [B] as a function of storage age.
Data are presented as the mean ± SD of ten sets of paediatric packs. ANOVA with post-ANOVA Duncan's multiple comparison test confirmed significant differences ($p<0.05$) between all points analysed with the exception of days 3 and 7.

Extracellular haemoglobin and malondialdehyde

In many studies of the storage of packed red blood cell units, the appearance of Hb in the extracellular medium is used as a marker of the degree of haemolysis. MDA is a robust marker of lipid peroxidation[1,22]. The changes in these parameters during storage is shown in Figure 2. Unlike the pattern of changes in iron and NTBI, both Hb and MDA rose more slowly over days 3 to 21 and then more steeply towards the end of the period of storage. The mean (± SD) total Hb level in the packs was 27.31±3.26 mg/dL. Assuming that extracellular Hb is a reasonable marker of haemolysis, the maximal level of Hb in the extracellular phase was 0.83% of the total.

Not surprisingly there were strong positive correlations between the level of oxidative damage (MDA) and Hb ($r=0.736$; $p<0.001$; $n=60$). In addition there were strong positive correlations between MDA and total iron ($r=0.785$; $p<0.001$; $n=60$) and MDA and NTBI ($r=0.602$; $p<0.001$; $n=60$).

Ascorbate and glutathione

Figure 3 shows how the level of total and reduced ascorbate varied according to the storage age of the units. Levels of both parameters remained relatively constant until around day 14 when they fell significantly to minimum levels on day 35. Comparing the data

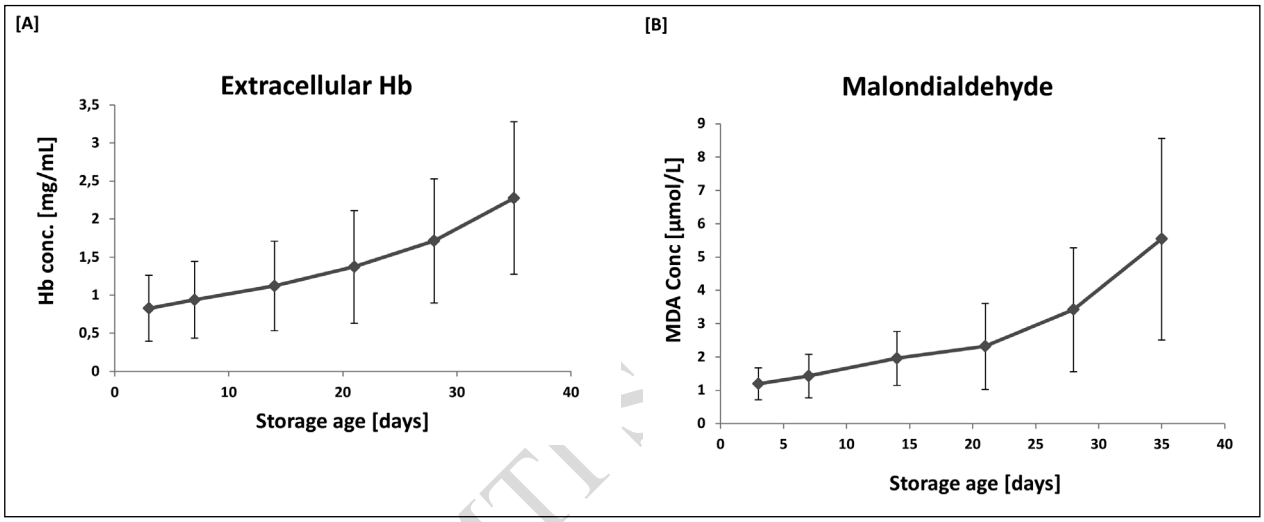

Figure 2 - Levels of extracellular Hb [A] and MDA [B] as a function of storage age.
Data are presented as the mean ± SD of ten sets of paediatric packs. ANOVA with post-ANOVA Duncan's multiple comparison test confirmed significant differences ($p<0.05$) between all points analysed in plot A (Hb) with the exception of days 28 and 35, and days 28 and 21. Similarly, in plot B (MDA) significant differences were observed between all points with the exception of days 28 and 14.

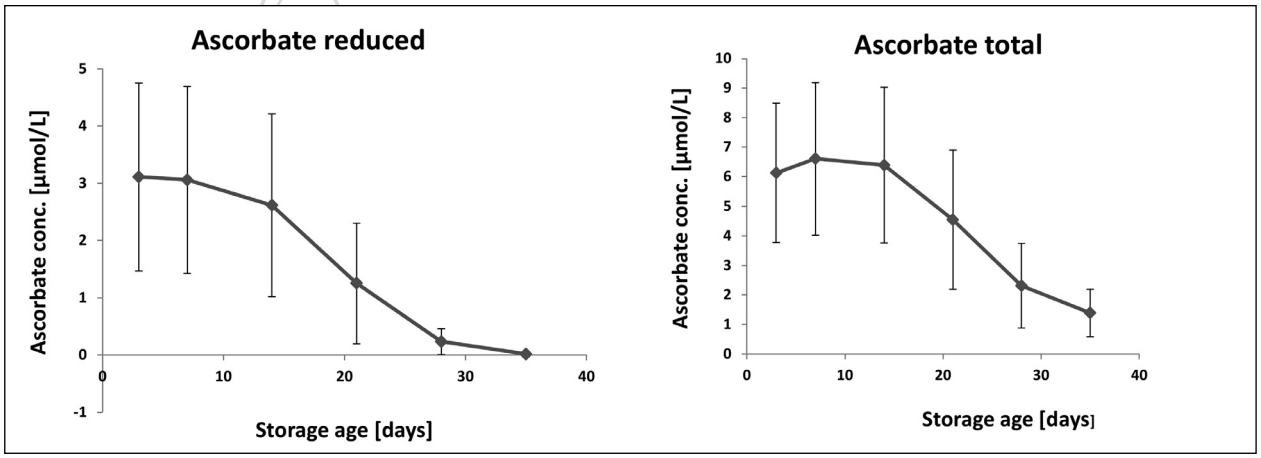

Figure 3 - Levels of extracellular total and reduced ascorbate as a function of storage age.
Data is presented as the mean ± SD of 10 sets of paediatric packs. Anova with post-anova Duncan's multiple comparison test confirmed significant differences ($P<0.05$) between levels on day 03 and days 21, 28 and 35, but not between days 3, 7 and 14 in both plots.

from the two graphs indicates that a high proportion of total ascorbate was in the oxidised form, with this amount reaching nearly 100% on day 35. The data also indicate that the ability to convert oxidised ascorbate back to the reduced form appears to be limited in stored packed red cells. 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[37]. It is possible that hydrolysis of dehydroascorbate during storage accounted for the loss of reducible ascorbate. The relationship between MDA and reduced and oxidised ascorbate showed a positive correlation between oxidised ascorbate and MDA ($r=0.465$; $p<0.01$; $n=60$), and a negative correlation between reduced ascorbate and MDA ($r=0.426$; $p<0.01$; $n=60$). Total iron and NTBI were positively correlated with oxidised ascorbate ($r=0.716$; $p<0.001$ [total iron]; $r=0.659$ $p<0.001$ [NTBI] $n=60$), and negatively with reduced ascorbate ($r=0.694$; $p<0.001$ [total]; $r=0.667$; $p<0.001$ [NTBI]; $n=60$).

The level of total glutathione was very low in the extracellular medium of the packed cell units and changed very little during storage (Table I). Furthermore, the addition of NEM to the samples during analysis effectively abolished the glutathione fluorescence indicating that, unlike the situation with regard to ascorbate, glutathione remains almost exclusively in the reduced form. In contrast, the level of GSH in whole blood was predictably high and decreased gradually from a maximum level on day 3 to a lowest value on day 35. These findings are similar to those reported previously[38,39]. In contrast to the previous studies, in this study a rise was not seen in GSSG in the later stages of storage. Over 90% of the GSH was in the reduced form. Oxidised GSSG changed little throughout the storage period. The magnitude of the loss of GSH over time in comparison to the level of GSSG indicates that in this study, the loss of GSH is more likely to be due to metabolism via a route other than oxidation to GSSG[40], coupled with reduced *de novo* synthesis in the latter stages of storage[38]. There was a strong negative correlation between total blood GSH and total extracellular iron ($r=0.723$ $p<0.001$; $n=60$) and NTBI ($r=0.691$ $p<0.001$; $n=60$).

Table 1 - GSH and GSSG levels as a function of storage age. Data is presented as mean level ± standard deviation of 10 observations. Anova with post-Anova Duncan's multiple comparison test confirmed significant differences in total blood glutathione ($p<0.05$) between the level at day 3 and all other days with the exception of day 7.

Storage age [days]	Total blood glutathione [µmol/L]	Total blood oxidised glutathione [µmol/L]	Extracellular glutathione [µmol/L]
03	621.10±64.56	5.700±1.318	0.709±0.467
07	581.11±48.60	5.253±0.992	0.713±0.376
14	570.09±56.05	4.995±2.182	0.532±0.176
21	520.28±39.21	4.615±1.516	0.521±0.196
28	480.39±39.38	4.799±1.671	0.525±0.178
35	442.79±37.78	3.494±0.952	0.574±0.231

Discussion

The results of this study have confirmed and extended a number of findings from previous work[24-28,41]. In addition, the combination of parameters measured in this study have provided data from which a potential mechanism may be proposed to account for the changes observed in paediatric packed red blood cells during storage. The presence of iron and NTBI in the extracellular medium surrounding the packed red blood cells on arrival at the blood bank, coupled with the presence of MDA and Hb indicates that initial changes occurring during blood collection and processing lead to oxidative damage to components of the packed cell units. This provides an initial pro-oxidant environment around and, probably, within the red blood cells. Following on from the initial damage to the erythrocytes, oxidative damage is on-going and progressive throughout the period of storage. The very high level of iron and NTBI in the extracellular phase indicate that it could not all have originated from the relatively low level of Hb present in the extracellular environment surrounding the red blood cells. It must have derived as a result of damage to iron-binding proteins within the red blood cell and leaked out into the extracellular fluid. Hb within the red blood cell is usually well protected by an array of antioxidants and other protective molecules[42]. When red blood cells are stored under refrigerated conditions the mechanisms protecting the cells lose their effectiveness and Hb becomes vulnerable to oxidation[43]. This has been confirmed in many previous studies[44]. In this study and others[38,39,45], the intracellular GSH level fell consistently throughout the period of storage and the extracellular level of MDA rose, indicating that oxidative damage to membrane phospholipids was ongoing. GSH is also an important anti-oxidant which protects haem and Hb against excessive oxidation[46], and the loss of intracellular GSH during storage would limit this degree of protection. Oxidative modification of Hb may include damage to the haem moiety at any of the double bonds which would lead to the release of free iron which may then participate in further generation of reactive oxygen species[47]. The free iron, and iron bound to haem may then be able to leave the red blood cell and accumulate in the extracellular medium of the stored packs. This scenario is likely as a variety of oxidising

agents have been shown to cause the release of iron from erythrocytes[13,14], and this appears to be associated with the formation of methaemoglobin, a relatively unstable Hb derivative which can readily release 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. The nature of NTBI in plasma is not fully understood[48], and even less is known about the nature of the NTBI in the extracellular fluid of stored packed cells. It is possible that a portion of the NTBI measured is iron bound to or removed from haem during the assay procedure. Previous studies in stored adult packs[49] showed that sufficient haem could be present in the extracellular fluid to contribute significantly to the measured total iron and NTBI levels. Further studies are required to find out more about the nature of the iron in these packs.

Previous studies also proposed that oxidative stress during storage drives many of the changes in protein and lipid structure that occur during storage[39,45]. Previous studies indicated that iron-induced free radical generation is capable of initiating lipid peroxidation and causing haemolysis in stored and circulating red blood cells[50-52]. Studies have shown positive effects of some anti-oxidants and iron chelators in defending against lipid peroxidation in stored erythrocytes[27,28], and fresh erythrocytes challenged with free radicals in vitro[53]. Ascorbate is particularly adept at defending against oxidative damage to the haemoglobin molecule[54]. Ascorbate is able to reduce the products of Hb oxidation and protect against the consequences of Hb oxidation such as the release of redox active iron[54]. The ability of ascorbate to reduce methaemoglobin is particularly important[55], as is its ability to reduce ferryl Hb[54] which is another derivative of Hb formed when Hb is oxidised[15]. The relative paucity of ascorbate, and the high degree of ascorbate oxidation would limit the protective action of ascorbate in packed cell preparations. Interestingly, a recent study demonstrated that the addition of ascorbate to stored murine red blood cells improved post-transfusion recovery of the red blood cells[45].

In this study we used extracellular Hb as a marker of haemolysis[35,56]. Comparing the measured total Hb and extracellular Hb , and assuming a haematocrit of around 60% (the target haematocrit during preparation of the packs), a calculated figure for the degree of haemolysis even at 35 days storage was only 0.33%. This is very close to the value reported by Gevi *et al.*[38], and well below the maximum of 0.8% recommended by the European guidelines. This further supports the view that the high level of extracellular iron and NTBI did not originate as a result of haemolysis but through intracellular oxidative damage, as outlined above. It is likely that some of the measured Hb is present in Hb-rich microparticles which are shed from the red blood cells during storage[57,58]. The proportion of Hb present in microvesicles during storage can be large and even exceed free extracellular levels[57]. These microparticles also contain other proteins and lipids, some of which may be oxidatively modified[59]. The purpose of the generation of microvesicles is unclear, but the Hb contained within them could contribute to the measured Hb in this study and be a source of free iron if oxidatively modified. Whether the Hb contained within microparticles can be considered as a valid marker of haemolysis is unclear. Consequently, the calculated degree of haemolysis based on extracellular Hb as measured in this study may be an overestimate. Importantly, the large build-up of iron and NTBI in the extracellular environment during storage occurs despite limited haemolysis.

The question that needs to be addressed is whether or not the changes in iron and other factors during storage might have a detrimental effect on the recipients. To put the data in context, total iron rose to about 30 μM and NTBI to about 15 μM in this study. Studies in our laboratory in adult controls and type 2 diabetics provided figures of total iron of 19±6.6 μM (controls, n=24) and 17.9±7.6 μM (diabetics, n=54). The median NTBI was 0.34 μM (range, 0-2.08) in controls and 0.21 μM (range, 0-1.34) in diabetics. Consequently, the iron status of the stored packs, particularly NTBI, is massively in excess of the normal values. Given that premature babies receive approximately 10% of their blood volume over a 4-hour transfusion period, the potential for iron and oxidative overload is significant, particularly since they are poorly equipped to deal with additional iron and oxidative load[18-20,60] being limited in the processes which operate to sequester free iron such as transferrin, caeruloplasmin and albumin. Premature babies also have limited anti-oxidant defences to protect against circulating free radicals[1,20,21,61]. Furthermore, the concentration of the low molecular weight anti-oxidants ascorbate, urate and possibly glutathione in serum and bronchoalveolar lavage fluid in premature babies falls during the first week of life and recovers over the next few weeks[1,21,61-63]. 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 anti-oxidant protection is falling. Consequently, the premature baby may be particularly susceptible to adverse reactions of transfused iron because of their poor anti-oxidant status and inability to deal with any excess iron. In addition, any free Hb or haem present in the transfused blood, and supplemented by post-transfusion haemolysis[64] would be potentially toxic in its own right[65,66] unless it is rapidly

sequestered by haptoglobin[65,67] and hemopexin[68]. The removal of plasma and the replacement with additive effectively removes most of the haptoglobin and hemopexin such that free Hb and haem could be available in the transfused blood. Premature babies appear to have haptoglobin in the circulation[69] and may be able to up-regulate the level as necessary[70]. Thus the potentially toxic influence of free Hb may be limited by the presence of haptoglobin in the recipients. In contrast, hemopexin levels are low[71] in premature babies who could be at risk of haem-mediated toxicity.

This study has highlighted potential mechanisms for storage-related changes in iron and oxidative load in paediatric packed red blood cell units. There is evidence and logic to suggest that these changes could be potentially dangerous if older stored blood were to be transfused to premature babies. However, these are effectively just theoretical potential risks and it is generally considered that there is no urgency to change transfusion practice in the neonatal intensive care unit.

This view is supported by the results of a recent randomised controlled study which examined the influence of blood stored for less than 7 days compared to that administered according to standard practice[72]. The mean storage age of the "fresh" blood was 5.1 days and that of the "older" blood 14.6 days. The study showed no changes in outcome with regard to the major consequences of prematurity *viz* necrotising enterocolitis, ROP, bronchopulmonary dysplasia and IVH as well as death. Our data would suggest that any major impact of iron and/or oxidative load in premature babies is likely to occur following transfusion with blood that has been stored in excess of 21 days and possibly more than 14 days.

This is supported by other studies which indicated that, from biochemical and molecular standpoints (in addition to iron and related factors), the parameters defining the integrity of SAGM-stored leucoreduced red blood cells (as studied here) may be acceptable up to 14 days of storage, but then decline[39]. For this reason, it may be pertinent to study the clinical impact of blood stored for longer than 14-21 days.

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