

TITLE PAGE

**The Red Cell Storage Lesion and Therapeutic Blood Transfusion in
the Critically Ill Patient.**

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DECLARATION

This thesis is entirely my own composition and has not been accepted or submitted for any other degree or professional qualification. The described work was performed by myself, except where specifically quoted in the text.

DEDICATION

This thesis is dedicated to my family:

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List of abbreviations

Abbreviation	Full text
ADP	Adenosine diphosphate
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BTS	Blood Transfusion Service
CABG	Coronary artery bypass graft
CaO ₂	Oxygen content of arterial blood
CI	Confidence interval
CO	Cardiac output
COHb	Carboxyhaemoglobin
CO ₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CPD	Citrate-phosphate-dextrose
cpm	Counts per minute
⁵¹ Cr	⁵¹ Chromium

CV	Coefficient of variation
DO ₂	Oxygen delivery
2,3 DPG	2,3 Diphosphoglycerate
ECG	Electrocardiogram
EDTA	Ethylenediamine tetra-acetic acid
fL	Femtolitres (1×10^{-15} L)
GAPD	Glyceraldehyde 3 phosphate dehydrogenase
G3P	Glyceraldehyde 3 phosphate
Hb	Haemoglobin
HEPES	Hydroxyethyl piperazineethanesulfonic acid buffer
ICU	Intensive care unit
KPa	Kilo-Pascal
MBq	Mega-Becquerel
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
O ₂ ER	Oxygen extraction ratio

OHEC	Oxygen haemoglobin equilibration curve
P_{aO_2}	Arterial partial pressure of oxygen
PBS	Phosphate buffered saline
PCO_2	Partial pressure of carbon dioxide
PGA	Phosphoglycerate
PGK	Phosphoglycerate phosphokinase
PGM	Phosphoglycerate mutase
P_i	Inorganic phosphate
PO_2	Partial pressure of oxygen
P_{50}	The PO_2 at which the Hb is 50% saturated with oxygen
pg	Pico-gram (10^{-12})
pHi	Gastric intramucosal pH
PS	Phosphatidylserine
PVC	Polyvinyl chloride
RBC	Red blood cell
RCC	Red cell concentrate
RCTT	Red cell transit time

Rh	Rhesus
rHuEPO	Recombinant human erythropoietin
rpm	Revolutions per minute
SAGM	Saline-adenine-glucose-mannitol
SaO ₂	Arterial oxygen saturation
SHOT	Serious Hazards Of Transfusion
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SNBTS	Scottish National Blood Transfusion Service
SO ₂	Oxygen saturation
^{99m} Tc	^{99m} Technetium
TCA	Trichloroacetic acid
TMB	Tetramethylbenzidine
TRALI	Transfusion related acute lung injury
vCJD	variant Creutzfeldt-Jakob disease
VO ₂	Oxygen consumption
WB	Whole blood

WBC White blood cell

1 Abstract of Thesis

1.1 Background

Anaemia is a common finding in critically ill patients. Currently, the transfusion of stored blood is the only treatment available to most patients. Despite this reliance on blood transfusion there is a marked lack of data about both the efficacy of red cell transfusion products and the clinical situations in which they are likely to be effective. It has recently been suggested that red blood cell (RBC) transfusions may have detrimental effects in critically ill patients and that these effects may be related to the transfusion of stored RBCs in particular. It is well recognised that RBCs undergo many metabolic and structural changes during refrigerated storage, these changes are termed the red cell storage lesion. The clinical implications of the red cell storage lesion are not known.

1.2 Aim

To assess the implications of the red cell storage lesion of the current UK RBC product, namely leucodepleted RBCs stored in saline-adenine-glucose-mannitol additive solution, using a combination of in vitro and in vivo studies.

1.3 Methods

1. The quality of the current RBC product was assessed using in-vitro assays of RBC oxygenation/de-oxygenation, namely P_{50} and the 2,3 diphosphoglycerate concentration, and RBC deformability.
2. Radiolabel studies were performed to determine the 24 and 48-hour recovery of stored allogeneic blood in critically ill patients.
3. The in-vivo regeneration of red cell 2,3 diphosphoglycerate (2,3 DPG) in stored blood transfused to critically ill patients was investigated.

4. Antigenic differences between donor and recipient were used to track allogeneic RBCs following therapeutic transfusions to determine RBC survival using a non-radioisotopic technique.

1.4 Results

1. In-vitro tests showed that current collection processing and storage procedures:
 - (a) Result in a very rapid reduction in red cell 2,3 DPG concentration. Approximately 50% of 2,3 DPG had been lost by day 2 of storage and it was barely detectable by day 14. The in-vitro P_{50} also decreased rapidly during storage; the time-frame of the decrease matched that of the decrease in 2,3 DPG.
 - (b) Result in a slight reduction in red cell deformability.
2. The current red cell product, stored for between 10 to 29 days, had a mean 24-hour recovery of 91% in critically ill patients.
3. Following transfusion to critically ill patients stored blood rapidly regenerated 2,3 DPG.
4. Red cell antigens were used to track allogeneic red cells for up to 12 weeks post-transfusion. The estimated median red cell lifespan was 104 days (range 86 to 124 days).

1.5 Conclusions

Current red cell storage methods fail to maintain red cell 2,3 DPG and result in a loss of red cell deformability. Although 2,3 DPG regeneration was found to occur rapidly it still took 24 to 72 hours for levels to approach normal; whether or not this is clinically significant is not known.

The current UK red blood cell product has good short-term and long-term survival characteristics following therapeutic transfusion.

1.6 Keywords:

Red cell storage lesion

Erythrocyte transfusion

Critical illness

2,3 diphosphoglycerate

Differential agglutination

Post transfusional recovery

Red blood cell survival

2 Brief History of Red Blood Cell Transfusion.

From ancient times blood has been regarded as a living substance and synonymous with life itself. The ancient Greeks and Romans considered blood to be the seat of the soul and to hold within it the physical and mental powers of the individual. Although blood letting (to purge poisons from the blood) was common practice in medieval times the administration of blood for acute haemorrhage seems not to have been considered. The history of blood transfusion is considered to start with William Harvey's discovery of the circulation of blood.

1628 William Harvey published '*An Anatomical Study of the Motion of the Heart and of the Blood in Animals,*' in which he expressed his view that blood was circulated in a closed system of arteries and veins by the pump action of the heart. Harvey's views were very controversial and lost him many patients; most people of the day believed that food was converted into blood by the liver and that blood in turn was consumed as fuel by the body. Nevertheless, Harvey's work undoubtedly laid the foundations of modern cardiovascular medicine.

Shortly afterwards, the earliest known blood transfusion was attempted and experiments soon began on the intravenous injection of various fluids. The first well-documented work was performed in 1656 by Sir Christopher Wren who used the quills of bird feathers and special bladders to infuse medicinal liquors such as opium, wine and ale into dogs.

1665 Physician Richard Lower performed the first successful transfusion at Oxford, transfusing blood from an artery of one dog into a vein of a second dog.

1667 In Paris, Jean-Baptiste Denis appropriated Lower's techniques and applied them to human transfusion. Denis infused approximately 9 oz (approximately 250 ml) of sheep's blood into a 15-year-old boy who was in an exhausted state following repeated venesections to treat a fever (an early example of severe iatrogenic anaemia). The boy made an excellent recovery and Denis was encouraged to perform further transfusions. Denis's success at

transfusing sheep's blood into humans owed much to the small volume he infused and sheer good fortune.

A prolonged debate took place as to who should be credited with the first blood transfusion. The question of priority was finally resolved by giving Lower credit for the first successful animal blood transfusion and Denis credit for the first successful blood transfusion in humans.

1678 Following several reports of severe transfusion reactions the Royal Society of England and the Paris Society of Physicians imposed a moratorium on transfusion practice prohibiting any further attempts involving humans.

The moratorium resulted in an almost total cessation of transfusion research for the next 150 years. Interest was not renewed until the late 18th century, following the discovery of oxygen by Joseph Priestley in 1777 and the demonstration of its role in respiration by Antoine Lavoisier in 1780. These developments led to an appreciation of the dangers of acute blood loss.

1818 James Blundell, a British obstetrician, recognised the need for a practical blood replacement therapy for the management of puerperal haemorrhage. Blundell performed the first successful transfusion of human blood to a patient for the treatment of postpartum haemorrhage. Using a syringe, Blundell extracted approximately four ounces (approximately 110 ml) of blood from the husband and successfully transfused the wife. Between 1825 and 1830 he performed 10 transfusions, five of which proved beneficial to his patients, and he published these results. Considering that he knew nothing of blood group compatibility and did not practice asepsis, his five successes were impressive and lucky.

More widespread acceptance of human-to-human blood transfusion was hindered by practical difficulties, in particular, coagulation, transfusion reactions and infection.

1835 Theodor Bischoff demonstrated that when the whole blood of one animal was injected into another species, toxicity and death resulted. Bischoff found, however, that the injected animal would tolerate defibrinated blood.

1840 At St. George's School in London, Samuel Armstrong Lane, aided by Dr. Blundell, performed the first successful whole blood transfusion to treat haemophilia.

1867 English surgeon Joseph Lister used antiseptics to control infection during transfusions. His work was prompted by Louis Pasteur's demonstration that infection was caused by fungi and bacteria.

1869 Adolf Creite, a medical student in Gottingen, showed that serum proteins had the property of both "dissolving" and bringing about "clustering" of red cells (lysis and agglutination in modern terminology).

1875 Another German, Leonard Landois, found more evidence of red cell agglutination and lysis. He performed in vitro experiments where he mixed red cells from one animal with serum from another, such as a lamb and a dog, and found that it produced agglutination and lysis. He also performed in vivo xenotransfusion studies in animals and noted that these resulted in haemolysis, widespread haemorrhage, haemoglobinuria, renal failure and death.

1890 Nicolas Maurice Arthus, a French physician & physiologist, and Calixte Pageo discovered the anticoagulant effect, attributable to the chelation of calcium, of the soluble sodium salts of oxalic and citric acids.

1900 Karl Landsteiner, an Austrian physician, discovered the first three human blood groups, A, B, and C. Blood type C was later changed to O. His colleagues Alfred Decastello and Adriano Sturli added AB, the fourth type, in 1902.

1907 Ludvig Hektoen, a US medical scientist, suggested that the safety of transfusion might be improved by cross-matching blood between donors and patients to exclude incompatible

mixtures. Reuben Ottenberg, a physician at Mount Sinai Hospital, New York performed the first blood transfusion using blood typing and cross-matching and over the next several years successfully used the procedure in 128 cases, virtually eliminating transfusion reactions. Ottenberg also observed the Mendelian inheritance of blood groups and recognized the "universal" utility of group O donors.

1912 Roger Lee, a US physician, demonstrated that it was safe to give group O blood to patients of any blood group, and that blood from all groups could be given to group AB patients. The terms "universal donor" and "universal recipient" were coined. His findings, coming shortly before World War I led to the use of Group O individuals as universal donors.

1914 Albert Hustin, of Brussels, reported the successful use of sodium citrate and glucose as an anticoagulant for blood removed from a patient one month previously and stored at 4°C before reinfusion.

1915 Dr Richard Lewisohn, at New York's Mount Sinai Hospital, formulated the optimum concentration of sodium citrate (2%) that could be mixed with donor blood to prevent coagulation whilst avoiding hypocalcaemia in the transfusion recipient.

1916 At the Rockefeller Institute in New York Francis Peyton Rous and J.R. Turner introduced a citrate-glucose solution that permitted the storage of blood for several weeks after collection.

Allowing for blood to be stored in containers for later transfusion aided the transition from the vein-to-vein method to indirect transfusion. These discoveries allowed for the establishment of the first blood depot by the British during World War I.

After the end of World War I interest in blood storage seems to have evaporated and it revived only in the 1930s. This was in part due to the decline in demand and also due to various untoward reactions attributed at the time to citrated blood (now thought to be due to

bacterial exotoxin contamination arising from the failure to use pyrogen free distilled water to make citrate and saline solutions).

1932 The first blood bank was established in a Leningrad hospital. By the mid-1930s, the Soviet Union had set up a national network of blood centers capable of storing blood, in cans, and distributing it to all corners of the country.

1937 News of the Soviet experience travelled to America, where Bernard Fantus, director of therapeutics at the Cook County Hospital in Chicago, established the first hospital blood bank. Fantus is recognised to have originated the term "blood bank."

1939/40 The Rhesus (Rh) blood group system was discovered by Karl Landsteiner, Alex Wiener, Philip Levine, and R.E. Stetson.

1939 The advent of World War II led to a massive increase in the demand for blood, which in turn led to the establishment of national programs for blood collection.

At first a simple solution of trisodium citrate was used for blood storage but then work started in an attempt to find better storage solutions.

Sugars were tested in the hope that, since RBCs were thought to be impermeable to them, they might act like colloids and protect against cell lysis. Although sucrose and dextrose were equally effective in the prevention of lysis during storage a solution of dextrose was recommended for the storage of human blood. This was a rather fortuitous decision because dextrose was later shown to have a beneficial effect of RBC metabolism whereas sucrose does not. RBCs metabolise dextrose to provide energy in the form of adenosine triphosphate (ATP) and to produce glycolytic intermediates such as 2,3 diphosphoglycerate (2,3 DPG).

Acidified solutions were known to diminish the rates of potassium efflux from RBCs and of lysis, however there was a reluctance to use them in clinical practice because it was thought that they might be harmful. The incentive to test acidified solutions for clinical use arose

from the fact that there was one major drawback of using solutions of trisodium citrate and dextrose, when they were autoclaved together substantial caramelization occurred and this was thought to be undesirable. It was known that when acid-citrate-dextrose (ACD) solutions were autoclave together little or no caramelization occurred. Since it was simpler to autoclave the entire preservative solution in the blood container rather than add them separately attention focused on using ACD solutions as the standard preservative.

1943 John Freeman Loutit and Patrick Mollison found that ACD solutions improved RBC viability during storage and therefore permitted longer storage, and had little effect on the recipient's acid-base balance. The composition of ACD is shown in Table 2-1.

1943 The first description of transfusion-transmitted hepatitis appeared in the literature.

Mid-1950s In response to the heightened demand created by open-heart surgery and advances in trauma care, blood use entered its most explosive growth period.

1959 Max Perutz of Cambridge University deciphered the molecular structure of haemoglobin.

1962 The polyvinyl chloride (PVC) bag, for the collection and storage of blood, was introduced. Until then blood had been collected into breakable glass bottles, but the introduction of PVC bags initiated modern blood banking. It allowed for the simple and sterile preparation of multiple blood components from a single donation collected into a sterile single-use container. Much of the credit for this development goes to Professor Carl Walter of Harvard Medical School.

It has subsequently been found that plasticisers (phthalates) in the PVC bag leach into blood during storage and appear to be incorporated into the RBC membrane. Although this is cause for concern there is no evidence of any adverse effects. In fact, the presence of plasticisers appears to improve both the in vitro and in vivo survival of stored RBCs.

Table 2-1 The composition of commonly used anticoagulant and additive solutions (g/100 mL if not otherwise stated)

	Anticoagulant solutions		Additive solutions	
	ACD	CPD	SAGM	Adsol 1
Citric acid (monohydrate)	0.8	0.327		
Tri-sodium citrate (dihydrate)	2.20	2.63		
Dextrose	2.46	2.32	0.818	2.2
Sodium dihydrogen phosphate		0.251		
Adenine			0.0169	0.027
Mannitol			0.525	0.75
Sodium chloride			0.877	0.9
Volume in container (mL)	67.5	63	100	100
pH	5.0	5.6	4.8	5.6
Osmolality (mOsm/kg)		438	369	

Key:

ACD Acid-citrate-dextrose

CPD Citrate-phosphate-dextrose

SAGM Saline-adenine-glucose-mannitol

1963 Baruch Blumberg discovered a novel antigen in the blood of an Australian aborigine. He labelled this antigen "Australian Antigen" and later identified it as the surface antigen of Hepatitis B virus.

1971 Hepatitis B surface antigen (HBsAg) testing of donated blood began.

1972 Apheresis was used to separate plasma and blood cells within an extra-corporeal circuit and in turn allow the selective removal of plasma or the cellular elements of blood.

The increasing demand for plasma, to be used mainly for the production of Factor VIII and albumin, led to the practice of harvesting plasma from freshly collected blood. Although it was recognised that packed (plasma reduced) RBCs retained viability during storage it soon became common practice to re-suspend the RBCs in an electrolyte media, or additive solution. Considerable work was done in an attempt to find the best electrolyte composition for RBC storage.

There was known to be a close association between the ATP content of stored RBCs and their viability. Adding adenosine to RBCs at the beginning of the storage period was found to maintain ATP and significantly improve RBC post-transfusional survival. Unfortunately adenosine has a number of undesirable effects (adenosine depresses sino-atrial and atrio-ventricular nodal activity, can cause hypotension and is metabolised to uric acid) and its clinical use could not be justified. However it was not long before adenine was identified as having all the beneficial effects of adenosine (ATP preservation and improved RBC viability) but without any of the adverse clinical effects.

Subsequently saline-adenine-glucose-mannitol (SAGM) solutions became the preferred additive solutions for the refrigerated storage of RBCs (mannitol was added to reduce in vitro cell lysis). The composition of SAGM is shown in Table 2-1.

Early 1980s With the growth of component therapy, the use of plasma fractionation products for coagulation disorders, and plasma exchange for the treatment of immune disorders and collection of blood components, hospital and community blood banks entered the era of transfusion medicine, in which doctors trained specifically in blood transfusion actively participate in patient care.

Blood component therapy and plasma fractions prepared from large pools of donor plasma provided the opportunities for the transmission of blood borne infections.

1981 The first case of Acquired Immune Deficiency Syndrome (AIDS) was reported.

1984 The virus, now known as Human Immunodeficiency Virus (HIV), was identified as cause of AIDS. It was identified simultaneously in France and the USA.

1985 The first blood-screening test to detect HIV was licensed and quickly implemented by blood banks to protect the blood supply.

1990 The first specific test for hepatitis C, the major cause of “non-A, non-B” hepatitis was introduced.

1996 HIV p24 antigen testing of donated blood was introduced in some centres to improve the detection of early phase HIV infection.

1999 New tests, utilising the polymerase chain reaction to amplify DNA/RNA sequences, were introduced to directly detect the genetic material of viruses like HCV and HIV.

1999 Universal leucodepletion of blood components was introduced in the UK as a precaution against the possible transmission of variant Creutzfeldt-Jakob disease (vCJD) by blood transfusion.

1999 The Transfusion Requirements In Critical Care (TRICC) study suggested that use of a liberal transfusion strategy to maintain the haemoglobin concentration >100 g/L in critically ill patients did not improve outcome.

2002 Transfusion was recognised as a route of transmission for the West Nile virus.

2004 Two cases of suspected blood-borne transmission of vCJD were reported in the UK.

For reference a summary of the characteristics of the different RBC products is shown in Table 2-2.

Table 2-2 Red blood cell products.

Red cell product	Introduction date	Storage time	Expressed as a % of that present in original donation			Additives	Contaminants
			Red cell content	Plasma content	WBC content		
Fresh blood (direct transfusion)	1818	Nil	As per donor whole blood			None	None
WB/citrate/glass bottle	WWI	24 hours	100	100	None removed	Trisodium citrate sol.	Leached from glass
WB/ACD/glass bottle	WWII	21 days	100	100	None removed	ACD	Leached from glass
WB/ACD/PVC bag	1960s	42 days	100	100	None removed	ACD	Plasticisers
Packed RBCs (plasma reduced)/PVC bag	1970s	42 days	100	15-30	None removed	ACD	Plasticisers
RBCs in additive solution e.g. SAGM	1970s	42 days	95-100	2-5	None removed	Additive solution	Plasticisers
Leucodepleted RBCs in additive solution	1999	42 days	90	2-5	99.99% removed (5×10^6/pack)	Additive solution	Plasticisers

Key: WB whole blood ACD acid-citrate-dextrose PVC polyvinyl chloride SAGM saline-adenine-glucose-mannitol

3 Anaemia and red blood cell transfusion in the critically ill patient.

3.1 Abstract.

Anaemia is a common finding in critically ill patients. There are often multiple causes. Obvious causes include surgical bleeding and gastrointestinal haemorrhage but many patients have no overt bleeding episodes. Diagnostic blood sampling can be a significant source of blood loss. In addition, critically ill patients have impaired erythropoiesis as a consequence of blunted erythropoietin production and direct inhibitory effects of inflammatory cytokines. The ability of a patient to tolerate anaemia depends on their clinical condition and the presence of any significant co-morbidity; maintenance of circulating volume is of paramount importance. There is no universal haemoglobin transfusion threshold. Current guidelines for critically ill and perioperative patients advise that at haemoglobin values <70 g/L red blood cell transfusion is strongly indicated and at haemoglobin values >100 g/L transfusion is unjustified. For patients with haemoglobin values in the range 70 to 100 g/L the transfusion trigger should be based on clinical indicators. Most stable critically ill patients can probably be managed with a haemoglobin concentration between 70 to 90 g/L. Uncertainties exist concerning the most appropriate haemoglobin concentration for patients with significant cardio-respiratory disease.

3.2 Introduction.

There is no universal definition of critical illness but most clinicians use the term to mean an acute illness associated with organ failure. In the UK, patients with failure of one or more organs are managed in an Intensive Care Unit (ICU). Anaemia is a common complication of critical illness. In the ICU it is common practice to treat anaemia with the transfusion of allogeneic stored red blood cells (RBCs). About 40% of critically ill patients receive a RBC transfusion during their ICU admission despite the adoption of restrictive transfusion strategies.^{1,2} Wide variations in transfusion practice exist,³ reflecting doubts about the safety, efficacy and indications for blood transfusion. This review will examine the evidence that supports current transfusion practice, focusing on three areas:

- i. The prevalence and causes of anaemia in the critically ill patient.
- ii. The indications for RBC transfusion in the critically ill patient.
- iii. The efficacy of RBC transfusion.

3.3 Anaemia: Definition.

Anaemia is a condition characterized by a decrease in the oxygen carrying capacity of blood. The oxygen carrying capacity of blood is probably best estimated by the mass of circulating RBCs. Since red cell mass is not easily measured in the clinical setting the practical clinical definition of anaemia is based on the haemoglobin (Hb) concentration or haematocrit (Hct) of whole blood. The World Health Organization's definition of anaemia is an Hb concentration that is below the normal range; the normal Hb range is the distribution of Hb concentrations found in a representative, large group of individuals. Under most circumstances the Hb concentration is a good indicator of the red cell mass, but changes in the plasma volume may lead to discrepancies. For example, an increase in the plasma volume will decrease the Hb concentration, which may be interpreted as worsening anaemia, even though the red cell mass remains unchanged. The "anaemia of pregnancy" is a well-

known example; during pregnancy the red cell mass increases by almost 50% but the Hb concentration usually falls because the plasma volume increases by more than 50%. In surgical and critically ill patients fluctuations in the plasma volume often occur due to intravenous fluid resuscitation and increased capillary leak; this may make the Hb concentration an imprecise estimate of red cell mass in these patient groups.^{4,5}

3.4 The prevalence and course of the anaemia of critical illness.

The term “anaemia of critical illness” is used to describe anaemia that develops during a critical illness. Estimates of the prevalence of the anaemia of critical illness vary. This reflects the well-recognized differences in case-mix and illness severity that exist between ICUs within a country and between ICUs in different countries.

Anaemia often develops early in the course of a critical illness. Many patients are already anaemic on admission to the ICU. A recent epidemiological study of 146 western European ICUs (ABC study) found that 63% of critically ill patients had an Hb concentration <120 g/L on admission to the ICU.² A recent audit of transfusion practice at a major UK teaching hospital found that an Hb concentration <90 g/L occurred in 55% of all patients who stayed more than 24 hours in the ICU and it also occurred early, on the first and second ICU days in 52% and 77% of these patients respectively.⁶ Overall the Hb concentration was <90 g/L for 45% of all patient days.

Blood use also increases with increasing length of ICU stay. As stated earlier, approximately 40% of critically ill patients receive a blood transfusion and the transfusion rate rises to 73-85% in patients who stay more than 7 days in the ICU.^{2,7} It has been estimated that critical illness is associated with an average transfusion requirement of 0.34 units of red cell concentrate per day.⁸

A large audit in Scottish ICUs found that, at the time of ICU discharge, almost 90% of patients were anaemic (males <130 g/L; females <115 g/L) and approximately 50% had an

Hb concentration <100 g/L.⁹ It is not known how long this anaemia persists, or what effect it has on post-ICU recovery, functional status, quality of life or longevity.

3.5 The aetiology of anaemia in the ICU.

Anaemia can result from blood loss, decreased RBC production, increased RBC destruction or it may be functional (a misleadingly low haemoglobin concentration in the presence of a normal or even an increased red cell mass). All of these factors may play a role in the anaemia of critical illness (Table 3-1).

Table 3-1 Causes of a low haemoglobin concentration in critical illness.

- 1) Sources of blood loss:
 - a) Phlebotomy.
 - b) Surgical bleeding.
 - c) Gastrointestinal bleeding.
 - d) Extracorporeal renal support.
- 2) Reduced RBC lifespan.
- 3) Reduced erythropoiesis:
 - a) Blunted erythropoietin production e.g. inflammatory cytokines, acute renal failure.
 - b) Resistance to the action of erythropoietin.
 - c) Decreased iron availability.
- 4) Functional anaemia e.g. expanded plasma volume secondary to fluid administration.

3.5.1 Blood loss.

Blood loss prior to ICU admission clearly plays a role in the anaemia of critical illness. The ABC study found that patients admitted to the ICU after emergency surgery had the lowest mean Hb concentration on admission (108 g/L), followed by those admitted after elective surgery (110 g/L), trauma (115 g/L) and for medical reasons (119 g/L).² Nevertheless, of all patients with an admission Hb concentration <100 g/L, approximately 50% had no history of anaemia or of acute bleeding.

Diagnostic blood sampling contributes significantly to the anaemia of critical illness. Early studies found that, on average, a critically ill patient lost 1 to 2 units of blood through blood sampling during their hospital stay.^{10;11} Phlebotomy continues to be an important source of blood loss. The ABC study found that the volume of blood lost through blood sampling averaged 41mL per 24 hours.² Another study estimated that phlebotomy accounted for 30% of the total blood transfused in the ICU.^{7;7} Simple measures, such as returning the initial volume of blood taken to clear the arterial line, or the introduction of small volume collection tubes can significantly reduce diagnostic blood loss.^{12;13} Unfortunately the uptake of such measures is poor.¹⁴

Other sources of blood loss may be significant. Gastrointestinal bleeding due to stress ulceration is an important complication of critical illness. Risk factors include mechanical ventilation, coagulopathy and renal failure.¹⁵ However, the contribution from gastrointestinal bleeding is probably overstated.¹⁶ Gastrointestinal bleeding, including occult blood loss, is uncommon in critically ill patients, especially in those receiving ulcer prophylaxis.¹⁷⁻²⁰

Several studies have found a strong association between acute renal failure and the development of anaemia.^{2;7;16;21} The nature of this association is unclear but it may be related to the blood loss associated with renal replacement therapy, although other factors such as impaired erythropoietin production may be more significant.

3.5.2 Shortened RBC life-span

Complement activation, such as occurs in critically ill patients with systemic inflammatory response syndrome (SIRS) or sepsis, could potentially cause accelerated RBC destruction, although no evidence of intravascular haemolysis has been found.¹⁶ Several studies have reported finding reduced RBC deformability in patients with sepsis²²⁻²⁵ and a separate report has suggested that loss of RBC deformability is associated with reduced RBC viability.²⁶ However direct evidence to show that critical illness, and sepsis in particular, reduces RBC lifespan is lacking.

3.5.3 Impaired erythropoiesis

Audits of transfusion practice have consistently found that many, if not most, transfusions are given to patients who have a low Hb concentration in the absence of acute bleeding.^{1;2;21} In the past the suspicion has been that occult blood loss was probably responsible for a lot of this anaemia. However, it is now apparent that RBC production of critically ill patients is not normal. Several studies have demonstrated inappropriately low reticulocyte counts in anaemic critically ill patients.^{16;27} This suppression of the bone marrow response appears to be associated with the persistence of the inflammatory state. Several mechanisms may be involved, many of which are implicated in the anaemia of chronic disease. First, inflammatory cytokines such as tumour necrosis factor α , interleukin-1 and interleukin-6 have been shown to directly inhibit RBC formation.^{28;29} Elevated concentrations of these cytokines are frequently present in the circulation of septic critically ill patients.^{30;31} This inhibition of RBC formation can be overcome; the bone marrow of critically ill patients has been shown to be able to respond to the administration of high doses of recombinant human erythropoietin (rHuEPO).^{27;32} Second, inflammation may impair iron availability for erythropoiesis. The interpretation of iron indices in inflammatory states is difficult because serum ferritin is increased and serum transferrin is decreased as part of the acute phase

response.^{33;34} ICU patients typically have a low serum iron, total iron binding capacity, and serum iron/total iron binding capacity ratio but the serum ferritin concentration is normal or more usually elevated.^{34;35} It has been speculated that these alterations could be a primitive physiological response that benefits the host by depriving invading pathogens of nutritionally required iron. Whether or not this response also impairs iron availability for erythropoiesis is unclear. Third, critically ill patients have inappropriately low erythropoietin concentrations for the degree of anaemia.^{16;27;36;37} This blunted erythropoietin response is thought to result from inhibition of the erythropoietin gene by inflammatory cytokines.³⁸⁻⁴⁰

It is apparent that the anaemia of critical illness may have many aetiologies but current evidence suggests that the main determinants are whole blood loss, with phlebotomy accounting for a significant proportion of this, and a blunted erythropoietic response.

3.6 The indications for RBC transfusion.

Best practice would ideally be based on well-founded clinical indications for RBC transfusion. Defining such indications is essential in order to avoid unnecessary transfusions and equally to ensure that blood is not withheld when it could be of benefit. Except in emergency situations, such as major haemorrhage, the decision to transfuse RBCs should follow an appraisal of the risks of acute anaemia versus the risks of RBC transfusion.

3.7 The risks of acute anaemia.

3.7.1 Physiological response to anaemia.

Oxygen is carried in the blood in two forms, dissolved in plasma and bound to Hb. The oxygen content of arterial blood (CaO_2) is described using the equation:

Equation 3-1

$$CaO_2 = 1.34 \times Hb \times SaO_2 + (0.23 \times PaO_2) \quad (\text{mL/L})$$

Each gram of Hb binds 1.34 mL of O₂ when it is fully saturated. Hb is the haemoglobin concentration (g/L). SaO₂ represents the arterial oxygen saturation of Hb. The amount of oxygen dissolved in plasma is represented by 0.23 multiplied by the partial pressure of oxygen in arterial blood (in kPa).

In health, more than 98% of oxygen is transported by Hb and the amount of oxygen dissolved in plasma is negligible (Table 3-2). Anaemia results in a decrease in oxygen-carrying capacity of the blood.

Table 3-2 The relative influence of anaemia on the oxygen carrying capacity of arterial blood.

Parameter	Normal	Anaemic	Anaemic + oxygen therapy.
Inspired oxygen (%)	21	21	100
Arterial partial pressure of oxygen (PaO₂) (kPa)	12	12	85
Arterial oxygen saturation (SaO₂) (%)	98	98	98
Hb concentration (g/L)	150	75	75
Dissolved oxygen (mL/L)	3	3	19
Hb bound oxygen (mL/L)	197	98	98
Total oxygen content of arterial blood (CaO₂) (mL/L)	200	101	117

The amount of oxygen delivered to the whole body (DO_2) is the product of total blood flow or cardiac output (CO) and CaO_2 :

Equation 3-2

$$DO_2 = CO \times CaO_2 \quad (\text{mL min}^{-1})$$

Typical values for a resting 70 kg man are:

$$1000 = 5 \times 200 \quad (\text{mL min}^{-1})$$

From these equations, it is apparent that tissue hypoxia may be caused by a decrease in oxygen delivery resulting from decreases in blood flow (ischaemia), Hb concentration or arterial Hb saturation. At rest the average healthy individual consumes approximately 250 mL of oxygen every minute, this is called the oxygen uptake or consumption (VO_2). The ratio of oxygen consumption to oxygen delivery is called the oxygen extraction ratio (O_2ER).

Equation 3-3

$$O_2ER = VO_2/DO_2$$

The normal oxygen extraction ratio is 0.2 to 0.3, indicating that only 20 to 30% of the oxygen delivered to the capillaries is taken up by the tissues.

The body responds to the development of anaemia with a variety of physiological responses aimed at maintaining tissue oxygenation. The main responses are an increase in cardiac output and an increase in the oxygen extraction ratio.

Cardiac output increases during normovolaemic anaemia. This effect appears to be due, predominantly, to the reduction in blood viscosity.⁴¹ A reduction in blood viscosity decreases the resistance to blood flow, which facilitates left ventricular emptying. The net effect is an increase in stroke volume and cardiac output.

Increases in heart rate and/or myocardial contractility play a minor role in increasing the cardiac output of a normal heart in anaemia as long as normovolaemia is maintained or

unless the anaemia is very severe. This is clearly advantageous because of the increase in myocardial oxygen consumption associated with these two factors.

The second group of compensatory mechanisms attempt to match oxygen delivery to oxygen demand at the tissue level by allowing oxygen extraction to increase. At the systemic level, there is a redistribution of blood flow to areas of high demand, like the myocardium and the brain.⁴² Animal studies suggest that there are a number of mechanisms, at the microcirculatory level, that help to maintain tissue oxygenation. When blood flows along a blood vessel the RBCs tend to congregate in the centre, which results in a lower haematocrit in the blood that enters the side branches; this effect is known as “plasma skimming.” The “normal” capillary haematocrit has been estimated at approximately 8.5%.⁴³ During normovolaemic haemodilution the reduction of the systemic haematocrit is not followed by a proportional fall in capillary haematocrit; this implies some sort of regulation or attempt to maintain the capillary haematocrit.^{44;45} As a consequence, within the capillaries, red blood cell flux is maintained^{44;46} In addition, RBCs normally lose oxygen as they travel through the arterial tree but in the anaemic state the pre-capillary oxygen loss appears to be reduced.⁴⁷^{44;45;47} The net effect of all of these changes is a more efficient utilisation of the remaining red cell mass; RBCs circulate between the lungs and tissues faster, they lose less oxygen en route and they are directed to where they are needed most.

In chronic anaemia there is an increase in the concentration of 2,3 disphosphoglycerate (2,3 DPG) within the RBC. 2,3 DPG competes with oxygen to bind to Hb. An increased concentration of 2,3 DPG will decrease the affinity of Hb for oxygen (i.e. a right shift of the oxygen haemoglobin dissociation curve) and promote the offloading of oxygen to the tissues. Studies in critically ill patients have found both high⁴⁸ and low⁴⁹ concentrations of RBC 2,3 DPG. These apparently conflicting results may reflect the influence of other factors such as hypoxaemia and acid-base status on the concentration of 2,3 DPG within the RBC.

3.8 The concept of a critical haemoglobin concentration.

The physiological responses to normovolaemic anaemia maintain tissue oxygenation as the Hb concentration falls. Eventually a point is reached where cardiac output and oxygen extraction are maximal and cannot increase anymore. Further reductions in the Hb concentration will lead to a decrease in oxygen delivery, and consequently a fall in oxygen consumption. This point is called the “critical DO_2 ,” it is the point at which energy production in cells becomes limited by the supply of oxygen i.e. oxygen consumption is supply dependent. The Hb concentration at which the critical DO_2 is reached is called the “critical Hb concentration.” It is important to realize that the critical DO_2 is not a fixed value, but varies between organs and is dependent on the metabolic activity of the tissue.

Studies in dogs,⁵⁰ pigs⁵¹ and baboons⁵² have demonstrated this critical Hb concentration to be around 40 g/L.

A series of studies of acute normovolaemic haemodilution in healthy volunteers and surgical patients have attempted to define critical oxygen delivery in humans.⁵³⁻⁵⁵ The initial study focused on the cardiovascular and metabolic response to acute normovolaemic haemodilution.⁵³ Aliquots of blood (450-900 mL) were removed to reduce the Hb concentration to 50 g/L. Normovolaemic was maintained with 5% human albumin and/or autologous plasma. At an Hb concentration of 50 g/L heart rate, stroke volume, and cardiac output were increased, and oxygen delivery was reduced. There was no evidence of inadequate oxygenation using global (whole body) indices: calculated oxygen consumption increased slightly from a mean of 3.07 to 3.42 mL kg⁻¹ min⁻¹ and plasma lactate concentration did not change. However the subsequent studies did find some evidence suggestive of organ-specific hypoxia. Continuous electrocardiographic (ECG) ST-segment analysis revealed that three of fifty-five subjects developed transient, reversible ST-segment depression at Hb concentrations of 50-70 g/L.⁵⁴ All three subjects were asymptomatic. Two

of these three subjects had significantly higher heart rates than those who did not have ECG changes at the same Hb concentrations. The investigators concluded that these ECG changes were suggestive of myocardial ischaemia and that the higher heart rates that developed during haemodilution may have contributed to the development of an imbalance between myocardial oxygen supply and demand. A later study focusing on cognitive function during acute normovolaemic haemodilution also found evidence suggestive of tissue hypoxia.⁵⁵ Acute reduction of the Hb concentration to ≤ 60 g/L produced subtle, reversible increases in reaction time and impaired immediate and delayed memory; no such changes were detectable at a Hb concentration of 70 g/L.

Such studies are useful in defining the critical DO_2 , and critical Hb concentration in healthy conscious humans at rest, but they must be extrapolated to other situations with caution. Critically ill patients may have pre-existing medical conditions such as ischaemic and valvular heart disease that can impair the compensatory mechanisms for anaemia. In addition, critical illness itself can also impair the compensatory mechanisms and increase oxygen consumption (Table 3-3). This means that the critical DO_2 may vary widely between patients and within the same patient over time.

Nevertheless, there is considerable clinical evidence from Jehovah's Witness patients that suggests that acute anaemia is well tolerated under many circumstances.

One widely cited case report documents the management of an 84-year-old male Jehovah's Witness who underwent total gastrectomy.⁵⁶ Invasive monitoring was sited pre-operatively and this allowed oxygen consumption and oxygen delivery to be calculated throughout the perioperative period. Massive bleeding occurred at operation and the patient died 12 hours after surgery with an Hb concentration of 16 g/L. The critical DO_2 was found to be 4.9 mL $kg^{-1} min^{-1}$; the Hb concentration at this critical DO_2 was 40 g/L.

Other reports of clinical experiences with Jehovah's Witnesses suggest that, for many patients, mortality is only increased at very low Hb concentrations (<50 g/L) and that survival is possible at extremely low oxygen-carrying capacity (Hb concentration as low as 14 g/L).^{57;58}

Table 3-3 Clinical factors that may increase the critical haemoglobin concentration.

- 1) Reduced Oxygen Delivery.
 - a) Decreased Cardiac Output:
 - i) Pre-morbid disease e.g. ischaemic heart disease, valvular heart disease.
 - ii) Hypovolaemia e.g. increased capillary leak.
 - iii) Arrhythmias e.g. atrial fibrillation.
 - iv) Pulmonary Embolism.
 - v) Acute septic cardiomyopathy.
 - b) Hypoxaemia secondary to acute respiratory failure.
 - i) Acute Lung Injury (ALI)/Acute Respiratory Distress Syndrome (ARDS).
- 2) Increased Oxygen Consumption:
 - a) Pain, stress, anxiety.
 - b) Shivering.
 - c) Fever.
 - d) Severe infection.
 - e) Sepsis/Systemic Inflammatory Response Syndrome (SIRS).
 - f) Trauma.
 - g) Surgery.
 - h) Burns.
 - i) Adrenergic drug infusions.
 - j) Work of breathing e.g. during weaning.
 - k) Convulsions.

3.9 Transfusion thresholds.

The adequacy of any Hb concentration in a given clinical situation depends on whether a sufficient amount of oxygen is carried to the tissues to meet their metabolic requirements. In practice, it is difficult to reliably detect tissue hypoxia in euvoaemic critically ill patients unless it has become severe. There are no specific clinical signs of tissue hypoxia. Well-known signs, such as hypotension and oliguria, often indicate organ dysfunction but they are late sequelae. Bedside monitors, such as indirect calorimeters for measuring whole body oxygen consumption, are also of limited value (Table 3-4). There is no clear-cut threshold below which oxygen consumption can be said to be inadequate, and because the critical DO_2 varies between organs whole body oxygen consumption has poor sensitivity for individual organ hypoxia. Organ specific monitors such as gastric tonometry also have limitations (Table 3-4). Interpretation of measurements can be difficult because of uncertainties about the clinical relevance of an abnormal result. A practical approach to the assessment of tissue oxygenation relies on an assessment of the adequacy of oxygen delivery and the detection of anaerobic metabolism, namely lactic acidosis (Table 3-4). The attraction of this approach is that the measures of cardiac output and arterial oxygen saturation are more robust and provide valuable information about cardio-respiratory function. However, this approach is based on the premise that a low oxygen delivery predisposes to tissue hypoxia, but there is no clear-cut threshold of oxygen delivery at which hypoxia occurs and an elevated whole blood lactate concentration is not specific for tissue hypoxia.⁵⁹⁻⁶¹ Ultimately no single monitor can establish the need for RBC transfusion and the decision must be based on clinical judgment. This state of affairs has led to the adoption of transfusion thresholds based on the Hb concentration; a transfusion threshold is the Hb value at which transfusion will be indicated in the absence of other clinical signs or symptoms of anaemia. Historically, the widely accepted clinical standard was to transfuse patients when the Hb concentration dropped below 100 g/L, although there was a lack of evidence to support this criterion.

Table 3-4 Physiological parameters that can be used to assess the adequacy of tissue oxygenation in critically ill patients.

Parameter.	Comment.
Oxygen delivery.	
Cardiac output	The gold standard technique uses a thermodilution method and requires a pulmonary artery catheter, which is invasive. Less invasive methods, such as the oesophageal Doppler monitor, are available.
Arterial oxygen saturation	Continuous pulse oximetry is essential.
Tissue oxygenation.	
Whole body parameters.	
Oxygen consumption	Can be measured at the bedside by indirect calorimetry. There is no clear-cut threshold at which oxygen consumption can be said to be inadequate. Insensitive for single organ hypoxia.
Oxygen extraction ratio (O_2ER).	Requires a pulmonary artery catheter. Normal range 0.2 to 0.3. An $O_2ER >0.5$ with an adequate cardiac output has been suggested as an indication for transfusion. ⁶²
Whole blood lactate concentration	Normal value $<2 \text{ mmol L}^{-1}$. Hyperlactataemia $>4 \text{ mmol L}^{-1}$. Not specific for tissue hypoxia. ^{59-61;63}
Acidaemia	Not specific for tissue hypoxia. ⁶⁴

Organ specific parameters

ST-segment analysis	Sensitive and specific for myocardial ischaemia (hypoxia). Single organ hypoxia is not necessarily an indication for transfusion.
Gastric tonometry	The tonometer is a carbon dioxide (CO ₂) permeable silicone balloon affixed to the end of a nasogastric tube. It measures the partial pressure of CO ₂ (PCO ₂) in the gastric mucosa. Gastric PCO ₂ is then used to calculate the gastric mucosal pH (pHi) using the Henderson-Hasselbalch equation. A decrease in pHi indicates a reduction in mucosal blood flow. The clinical relevance of this measurement is still to be determined.

The formulation of subsequent guidelines has also been hindered by the lack of clinical evidence. Fortunately, we now have a high quality randomised controlled trial, the Transfusion Requirements In Critical Care (TRICC) trial⁶⁵ and a Cochrane review⁶⁶ of this and other smaller studies to guide us.

The TRICC trial investigated whether a restrictive approach to RBC transfusion that maintained the Hb concentration between 70 and 90 g/L was equivalent to a more liberal strategy of maintaining the Hb concentration between 100 and 120 g/L. Critically ill patients with a Hb concentration <90 g/L were randomly allocated to either the restrictive strategy group or the liberal strategy group. Patients who were actively bleeding were excluded from the study. RBC transfusions were administered when the Hb concentration fell below 70 and 100 g/L respectively. Patients received one unit of RBCs per transfusion. The study was stopped prematurely because of a low enrolment rate, which has been attributed to a

perception amongst physicians of a lack of equipoise within the 2 arms of the trial and the negative publicity generated by a high profile enquiry into “tainted blood” that was being conducted at the time. At completion 838 patients had been enrolled, which is only 52% of its target recruitment and the study was therefore underpowered. The two groups were very well matched in terms of gender, age and illness severity scores. The mean Hb concentrations after intervention were 107 g/L in the liberal strategy group and 85 g/L in the restrictive strategy group. The primary outcome, the 30-day all-cause mortality rate, was 18.7% in the restrictive strategy group and 23.3% in the liberal strategy group. Although this is an absolute difference of approximately 5% it did not reach statistical significance ($p=0.11$, 95% confidence interval for the difference between the groups, -0.84 to 10.2%). Cardiac complications, in particular new myocardial infarction and pulmonary oedema, were more common in the liberal strategy group ($p<0.01$). Pre-determined sub-group analyses found that the 30-day mortality rates were significantly lower with the restrictive transfusion strategy among patients who were less acutely ill (Acute Physiology And Chronic Health Evaluation II score ≤ 20) and among patients who were less than 55 years of age, but not among patients with clinically significant cardiac disease.

Other studies in orthopaedic,^{67;68} vascular⁶⁹ and low risk coronary artery bypass graft (CABG) patients^{70;71} found no difference in either morbidity or mortality outcomes between restrictive and liberal transfusion strategies but these studies were small and had inadequate analytical power to show significant differences in mortality or cardiac events.

The published evidence suggests that a restrictive transfusion strategy is at least as effective as and possibly superior to a liberal transfusion strategy in critically ill patients and provides compelling evidence that an Hb concentration in the 70 to 90 g/L range, is well tolerated by most critically ill patients. However, it should be noted that most of the data on clinical outcomes were generated by a single trial. Furthermore, there are concerns that some groups of critically ill patients, such as those with cardiovascular disease and patients who are

difficult to wean from mechanical ventilation, may benefit from higher Hb levels. These scenarios will be examined in more detail.

3.10 Ischaemic heart disease.

The concern that critically ill patients with ischaemic heart disease may require higher Hb levels arises from our understanding of myocardial oxygen kinetics, from animal studies that simulated coronary artery disease and anaemia, and clinical studies of patients suffering from coronary artery disease that subsequently underwent surgery or had a critical illness.

Myocardial oxygen consumption is directly related to the amount of work the heart performs. Therefore myocardial oxygen consumption is increased by tachycardia, increased afterload (hypertension), increased contractility and to a lesser extent by increased preload. Because the resting oxygen extraction ratio of the heart is near maximal (about 0.6) increased myocardial oxygen demand must be met by increasing coronary artery blood flow.⁵² This is achieved primarily by vasodilatation. Any restriction to vasodilatation, such as coronary artery disease can therefore limit coronary blood flow and myocardial oxygen delivery. From this brief review of myocardial physiology it can be seen that the risk of myocardial ischaemia (hypoxia) in a critically ill patient with pre-existing ischaemic heart disease depends not only on their degree of anaemia but also on their current cardiovascular status (heart rate, blood pressure and cardiac output).

There are several animal studies investigating the relationship between normovolemic haemodilution and various types of coronary artery stenosis.⁷²⁻⁷⁴ These confirm that coronary artery narrowing decreases the tolerance of the heart to anaemia, with exact values of Hb depending on the model used. It is difficult to extrapolate findings from these animal models to the clinical setting. As well as the inherent interspecies differences, most of the models were not subject to major fluctuations in myocardial work and most examined single coronary artery lesions rather than diffuse or multi-vessel disease.

There are a number of studies investigating the effects of anaemia in surgical patients with ischaemic heart disease or at high risk for it. In a case control study, continuous ambulatory ECG monitoring was performed on 27 high-risk patients undergoing infra-inguinal arterial bypass procedures.⁷⁵ ECG monitoring was started 12 hours preoperatively and continued for at least 48 hours postoperatively. Thirteen of 27 patients had a haematocrit <28%. Of these 13 patients, 10 demonstrated post-operative myocardial ischaemia (ST segment depression with or without chest-pain) and 6 sustained a morbid cardiac event (four had non-Q-wave myocardial infarctions, one unstable angina and one ischaemic pulmonary oedema). Only 2 of the 14 patients with a haematocrit >28% displayed myocardial ischaemia and none sustained a morbid cardiac event. In a similar study continuous ambulatory ECG monitoring was performed on 190 patients undergoing radical prostatectomy.⁷⁶ Tachycardia and a haematocrit <28% (measured immediately after surgery) were found to be independently associated with both intraoperative and postoperative ECG evidence of myocardial ischaemia.

Several large observational studies have confirmed the association between anaemia and cardiac morbidity and mortality in patients with ischaemic heart disease.

A retrospective study of 1958 patients who underwent major surgery and declined blood transfusion for religious reasons found that the relative risk of death associated with a low pre-operative Hb concentration (<100 g/L) was consistently higher in patients with cardiovascular disease than in patients without.⁷⁷

Similarly, an analysis of 4470 critically ill patients in Canadian ICUs found that in patients with cardiac disease there was a trend toward an increased mortality when Hb values were <95 g/L compared with anaemic patients with other diagnoses.⁷⁸

There is also evidence to suggest that the correction of anaemia may improve clinical outcome in patients with significant cardiac disease.

Anaemia is a recognised independent risk factor for congestive cardiac failure.⁷⁹ In a randomised controlled trial in 32 patients with severe congestive cardiac failure correction of mild anaemia (Hb concentration 100 to 115 g/L) with erythropoietin resulted in significant improvements in functional status.⁸⁰ There were four deaths in the control group compared to none in the treatment group, although this failed to reach statistical significance.

Support for an Hb transfusion threshold nearer 100 g/L for patients with severe ischaemic heart disease has come from a recent large retrospective cohort study in 79,000 patients aged >65 years with acute myocardial infarction.⁸¹ Patients with lower haematocrit values on admission had higher 30-day mortality rates. RBC transfusion was associated with a reduction in 30-day mortality among patients with an admission haematocrit <0.33 (less than about 110 g/L). No such association was observed for patients with an admission haematocrit >0.33.⁸²

In a retrospective analysis of the TRICC dataset the investigators identified a subgroup of 257 patients who were known to have ischaemic heart disease from ICU admission diagnoses and listed co-morbidity.⁸³ For these patients there were no statistically significant differences in all survival measures (30-day, 60-day, ICU and hospital mortality rates) but this is the only subgroup where there was a trend towards better outcome in patients whose Hb concentration was kept >100 g/L (p=0.3). However this was a retrospective subgroup analysis and should be interpreted cautiously.

These studies taken together suggest that an Hb transfusion trigger of 90-100 g/L may be more appropriate than lower Hb concentrations in patients with ischaemic heart disease. However, large prospective randomised controlled trials are required to confirm these findings and to explore the possibility that Hb values >100 g/L might confer benefit in specific patient groups.

3.11 Weaning from mechanical ventilation.

Weaning is the gradual withdrawal of mechanical ventilatory support. If weaning is to be successful the patient must perform the additional work of breathing that was previously being done by the mechanical ventilator. The majority of critically ill patients receiving mechanical ventilation can be weaned rapidly and easily. Failure to wean is often associated with respiratory muscle weakness or intrinsic lung disease. In patients with respiratory disease the work of breathing may be much higher than in patients with normal lungs and weaning can result in a significant increase in whole body oxygen consumption. It has been suggested that RBC transfusion may help anaemic patients cope with the increased oxygen demands of weaning. A case series describes 5 anaemic patients with chronic obstructive pulmonary disease (COPD) who had failed several trials of weaning from the ventilator.⁸⁴ Following transfer to a regional weaning centre the patients received RBC transfusions to increase the Hb concentration to 120 g/L or greater. Subsequently all the patients were weaned successfully. In a second study, the same investigators found that blood transfusion decreased minute ventilation and the work of breathing in moderately anaemic non-critically ill patients with severe COPD but not in anaemic patients without lung disease.⁸⁵ These are intriguing observations but further evidence is required.

In another retrospective subgroup analysis of the TRICC dataset 713 patients were identified who were receiving mechanical ventilation at the time of enrolment.⁸⁶ Three hundred and fifty seven patients had been randomised to the restrictive strategy group and 356 patients to the liberal strategy group. There were no significant differences in the duration of mechanical ventilation, in the number of ventilator-free days or in the time to extubation between the two groups. However as mentioned earlier there were significantly increased rates of myocardial infarction and pulmonary oedema in the liberal strategy group. There are a number of limitations to this analysis. The TRICC study was not designed to investigate the effects of RBC transfusion on weaning from mechanical ventilation, therefore weaning

algorithms were not used and it was not powered for this end-point. These points are particularly relevant because most of the study patients should have been capable of being weaned rapidly and easily.

Unfortunately there is insufficient evidence to draw any conclusions about the value of RBC transfusion in patients being weaned from mechanical ventilation. If RBC transfusion does facilitate weaning it is probably only significant in the small group of patients who have failed previous weaning attempts. Alternatively, it is also possible that RBC transfusion may have a detrimental effect on weaning from mechanical ventilation. Complications such as pulmonary oedema due to volume overload or an increased rate of nosocomial infections resulting from transfusion related immunomodulation could prolong the time a patient receives mechanical ventilation. In addition, it is possible that RBC transfusions may not improve oxygen delivery but may hinder the process because of the red cell storage lesion (see Section 3.13).

3.12 Risks of red blood cell transfusion.

Transfusion risks are the subject of numerous publications.⁸⁷⁻⁸⁹ Some risks are well documented and have been quantified (Table 3-5). Others, such as transfusion related acute lung injury (TRALI), transfusion related immunomodulation and the red cell storage lesion are poorly understood, but are potentially significant risks to the critically ill patient.

TRALI, reviewed elsewhere,⁹⁰ has a very similar presentation to acute respiratory distress syndrome (ARDS) and is probably significantly under-diagnosed in the critically ill population. However the distinction between ARDS and TRALI may be somewhat artificial. Most cases of ARDS have multiple risk factors and it has been postulated that, in some cases, TRALI may contribute towards the development of ARDS.⁹¹

Table 3-5 Transfusion related adverse events. The data are taken from the UK Haemovigilance programme (SHOT) annual report for 2000 to 2001.⁸⁹ Approximately 3.5 million blood components were issued during this period.

Transfusion related adverse event.	Number of events
Incorrect blood component transfused.	213
Delayed transfusion reaction.	40
Acute transfusion reaction.	37
Transfusion related acute lung injury.	15
Transfusion transmitted infection.	6
Post transfusion purpura.	3

There is now convincing evidence that non-leucodepleted allogeneic RBC transfusion has long-term immunosuppressive effects. This phenomenon was first described in renal transplant recipients⁹² but it is still clinically important even with modern immunosuppressive regimens.⁹³ The clinical significance of this effect in other settings is controversial. A number of studies have found an association between allogeneic RBC transfusion and increased rates of tumour recurrence.⁹⁴⁻⁹⁶ In addition, several studies have found an association between allogeneic RBC transfusion and an increased incidence of nosocomial infections such as pneumonia, wound infections and intra-abdominal sepsis.⁹⁷⁻¹⁰¹ This has led to growing concern that critically ill transfusion recipients may be predisposed to nosocomial infections, which may ultimately lead to higher mortality rates. The pathogenesis of transfusion related immunomodulation is poorly understood, but allogeneic leucocytes or leucocyte-derived bioactive mediators are thought to be involved.¹⁰² More

detailed discussions of transfusion related immunomodulation and the impact of universal leucodepletion can be found elsewhere.¹⁰³⁻¹⁰⁵

3.13 The red cell storage lesion and the efficacy and safety of blood transfusion.

The principal aim of blood transfusion is to augment the oxygen-carrying capacity of blood and thereby improve tissue oxygenation. Blood transfusion undoubtedly increases calculated oxygen delivery but the effect on tissue oxygenation and oxygen consumption is unclear.

RBCs undergo marked changes during refrigerated storage (Table 3-6); these changes are collectively termed "the red cell storage lesion." The red cell storage lesion may have a significant detrimental effect on RBC function and the efficacy of blood transfusion. After only 10 days of blood bank storage red cell 2,3 DPG is virtually undetectable.¹⁰⁶ This results in an increased affinity of Hb for oxygen and may impair the ability of RBCs to unload oxygen to the tissues. RBCs also undergo marked morphological changes during storage. These begin immediately after collection and consist largely of echinocytic change (the RBCs develop finger-like projections and adopt a spiky appearance).¹⁰⁷ This shape change is initially reversible but with increasing duration of storage it becomes permanent as the finger-like projections bud off to form micro-vesicles (RBCs lose approximately 25% of their membrane phospholipids during 42 days of storage).¹⁰⁸⁻¹¹¹ The net effect of these morphological changes is a decrease in RBC deformability. Such observations have led to suggestions that transfusing RBCs that are both 2,3 DPG depleted and poorly deformable could be ineffective and/or harmful. Two studies in particular are widely quoted as evidence of the significance of the red cell storage lesion. The first study investigated the effects of a 3-unit blood transfusion on oxygen kinetics in septic critically ill patients.¹¹² Transfusion had no effect on systemic oxygen consumption, the primary end-point. However retrospective analysis revealed an inverse association between the change in gastric mucosal pH (pHi), measured by gastric tonometry (see Table 3-4), and the storage age of the transfused blood.

Patients receiving non-leucodepleted blood that had been stored for more than 15 days had a decrease in pHi following RBC transfusion, which was interpreted as indicating worsening gastric mucosal oxygenation. The authors suggested that this could have been due to poorly deformable transfused RBCs causing microcirculatory occlusion. The second much-quoted study found that 28-day-old rat blood failed to improve systemic oxygen consumption in rats in contrast to fresh rat blood.¹¹³ However, these early findings have been challenged. A recent study found that rat RBCs deteriorate much more rapidly during storage than human RBCs and that after 28 days of storage only 5% remain viable.¹¹⁴ This obviously has major implications for animal models of transfusion.

Further contradictory evidence has been provided by a recent prospective, double blind, study in which stable ICU patients were randomised to receive fresh (median storage age 2 days) or “stored” (median storage age 28 days) leucodepleted red cell concentrates when the pre-transfusion Hb concentration was approximately 85 g/L.¹¹⁵ There was no evidence of any adverse effect following the transfusion of stored RBCs. In particular, there was on average no change in pHi or any measured index of oxygenation.

Although many clinical studies have attempted to define the impact of RBC transfusion on oxygen kinetics the considerable literature in this area is confusing because of varying methodology, differing patient groups and apparently contradictory findings. These issues are illustrated in a recent review that identified 14 studies on this subject.¹¹⁶ Blood transfusion consistently increased oxygen delivery but oxygen consumption increased in only 5 of the studies. Most of the studies, including the 5 that reported an increase, calculated oxygen consumption from pulmonary artery catheter derived measurements. Calculating oxygen consumption can introduce mathematical errors that couple oxygen delivery and oxygen consumption.^{117;118} This coupling may be erroneously interpreted as evidence of oxygen supply dependency. It is now recommended that oxygen

Table 3-6. The red cell storage lesion.

Red cell storage lesion:		
Metabolic changes		Potential clinical significance.
2,3 DPG depletion	2,3 DPG is virtually undetectable after 10 days of blood bank storage. ^{106;119}	2,3 DPG depletion results in a left shift of the oxygen Hb dissociation curve which may impair oxygen unloading to the tissues. Restoration of 2,3 DPG occurs within 24-72 hours in healthy volunteers ¹²⁰ , and in stable anaemic patients ^{121;122} but may take much longer in sicker patients. ^{123;124}
ATP depletion	ATP declines slowly during storage. ¹⁰⁶	Early studies found an association between the ATP concentration and post-transfusal survival although this is now contested. ¹²⁵

Structural changes

<p>Discocyte-Echinocyte-Spherocytinocyte shape change.¹⁰⁷</p>	<p>Initially reversible but becomes irreversible with increasing duration of storage.¹⁰⁷</p>	<p>The net effect of these changes is the loss of RBC deformability.</p>
<p>Membrane phospholipid loss</p>	<p>Microvesicle formation correlates with RBC shape change (see above) and results in a decreased RBC surface area to volume ratio.¹⁰⁸⁻¹¹¹</p>	<p>Loss of RBC deformability is associated with reduced post-transfusion viability.^{26;109} It has been postulated that poorly deformable transfused RBCs may cause microcirculatory occlusion.¹¹²</p>
<p>Loss of RBC deformability.</p>	<p>In vitro tests have documented loss of RBC deformability with increasing duration of storage.^{126;127}</p>	
<p>Loss of membrane phospholipid asymmetry</p>	<p>Phosphatidylserine (PS) accumulates in the outer membrane.¹²⁸</p>	<p>PS appearance in the outer membrane is thought to herald RBC clearance.</p>

consumption should be measured.¹²⁹ Furthermore, increasing oxygen delivery will only lead to an increase in oxygen consumption if oxygen supply dependency exists. The pre-transfusion Hb concentration in these studies ranged from 83 to 110 g/L, which is high compared to previous estimates of the critical Hb concentration. This questions the rationale for attempting to increase oxygen delivery by transfusion in mild to moderate anaemia.

On current evidence, the assumption that the transfusion of stored RBCs improves tissue oxygenation in anaemic critically ill patients is unproven.

3.14 Recombinant Human Erythropoietin in the critically ill.

The rationale for rHuEPO therapy is that increased erythropoiesis will result in higher Hb levels and subsequently reduce the need for RBC transfusions. This rationale was confirmed by early experimental studies demonstrating that rHuEPO given in the perioperative period accelerated erythropoiesis and resulted in significantly shorter times to return to baseline Hb levels.¹³⁰⁻¹³² The efficacy of perioperative rHuEPO has been demonstrated in a variety of elective surgical settings.¹³³⁻¹³⁵ Similarly, in critically ill patients with multiple organ failure rHuEPO therapy will also stimulate erythropoiesis. A prospective trial randomized 160 critically ill patients to receive rHuEPO or placebo.³² rHuEPO was given in a dose of 300 u/kg daily for 5 days and then on alternate days for a minimum of 2 weeks, or until ICU discharge, and a maximum of 6 weeks. The rHuEPO group was transfused with a total of 166 units of RBCs compared to 305 units transfused to the placebo group. Despite receiving fewer RBC transfusions patients in the rHuEPO group had a significantly greater increase in haematocrit. The same investigators have recently published the results of a much larger multicentre trial.¹³⁶ In this trial 1302 critically ill patients, who remained in the ICU for at least 2 days, were randomized to receive rHuEPO or placebo. rHuEPO was given in a dose of 40 000 units once a week for up to 4 weeks. Patients were also given oral iron therapy. The percentage of patients who received any RBC transfusion during the 28-day study-

period was significantly lower in the rHuEPO group than in the placebo group (50.5% vs. 60.4%, $p < 0.001$). This was despite similar transfusion triggers for the 2 groups (the mean Hb transfusion threshold was 85 g/L in each group). The reduction in the total number of RBC units transfused and the increase in Hb concentration were more modest in this study than the earlier trial. These differences may be accounted for by the shorter follow-up period and the smaller total dose of rHuEPO used in the second trial. Should rHuEPO be used in all patients admitted to the ICU for 3 or more days? Although rHuEPO treatment reduces RBC transfusions in critically ill patients further work is necessary to determine whether it improves morbidity and mortality. Also, rHuEPO is expensive (approximately £300 per 40 000 units i.e. one dose) and 10 patients had to be treated with rHuEPO to prevent 1 patient from receiving a transfusion during the 28-day study.

3.15 Conclusion.

Anaemia is a common finding in critically ill patients and it is now recognized to be a consequence of the critical illness itself. The pathogenesis of the anaemia of critical illness is multifactorial. Acute bleeding episodes, phlebotomy and impaired erythropoiesis are the main cause. Currently allogeneic RBC transfusion is the only therapy available to most patients. rHuEPO has been shown to reduce transfusion requirements but further evaluation is required. Potential RBC shortages and continuing concerns about blood safety mean that guidelines based on sound evidence of clinical effectiveness are urgently required. Data from one good quality randomized controlled trial (TRICC) provide a basis, but it is not known if the results can be applied to specific subgroups of patients, such as those with ischaemic heart disease or severe lung disease.

3.16 Summary:

- Anaemia is a common complication of critical illness.
- It is caused by impaired erythropoiesis and blood loss, such as surgical bleeding and phlebotomy.
- Many, if not most, RBC transfusions performed in the ICU are administered to treat anaemia rather than acute bleeding.
- Most stable critically ill patients, including those with mild ischaemic heart disease, can be managed with an Hb transfusion threshold of 70 g/L aiming to keep the Hb concentration between 70 to 90 g/L.
- Critically ill patients with severe ischaemic heart disease should probably have an Hb transfusion threshold nearer 90 to 100 g/L.
- The clinical significance of the red cell storage lesion is unclear.
- Recombinant human erythropoietin treatment has been shown to reduce transfusion requirements in critically ill patients.

4 The current red cell product and the red cell storage lesion.

4.1 Abstract.

Blood transfusion involves the transfusion of blood that may have been stored in a fridge for, in the UK, up to 35 days. RBCs undergo many changes during refrigerated storage; these changes are collectively termed "the red cell storage lesion." It has been suggested that universal leucodepletion of blood products may improve the quality of RBC storage by reducing RBC exposure to harmful cytokines. In addition, current quality parameters may not be the best predictors of RBC efficacy. Therefore, an assessment of the quality of the current red cell product, namely leucodepleted RBCs stored in saline-adenine-glucose-mannitol additive solution, was performed, focusing on in-vitro assays of red cell oxygenation/de-oxygenation and deformability; the study investigating the effects of refrigerated storage on RBC deformability is reported in Chapter 5. Ten blood donors, who met UK blood transfusion service donor criteria, were recruited. All of the leucodepleted RCC had a total Hb ≥ 45 g, which is well above the recommendation of >40 g. After 35 days of refrigerated storage very little haemolysis had occurred but there were marked metabolic changes; the pH of the RBC packs had decreased from 7.01 to 6.44, and RBC adenosine triphosphate concentration had fallen from 4.6 $\mu\text{mol/g Hb}$ to 3.3 $\mu\text{mol/g Hb}$. The RBCs rapidly became 2,3 diphosphoglycerate (2,3 DPG) depleted (14 and 0.7 $\mu\text{mol/g Hb}$ at 0 and 14 days respectively). There was a marked left-shift of the oxygen haemoglobin equilibration curve; the in vitro p50 decreased from 27.5 mmHg at the time of donation to 16.3 mmHg after 35 days. There was a very strong association between the RBC 2,3 DPG concentration and the in vitro p50.

Current UK RBC collection, processing and storage methods result in marked metabolic changes.

4.2 Introduction.

Blood transfusion involves the transfusion of blood that may have been stored in a fridge for, in the UK, up to 35 days. RBCs undergo many changes during refrigerated storage, such as 2,3 DPG depletion and a loss of deformability.¹³⁷ These changes are collectively termed "the red cell storage lesion." The implications of the red cell storage lesion for transfusion recipients have been debated for many years. A recent study in critically ill patients found that RBC transfusion was associated with a worse outcome.⁶⁵ The exact reasons for this are unclear but it has been postulated that the red cell storage lesion or the presence within the transfused blood of contaminating white blood cells (WBCs) may have played a role.

In the UK blood donations now undergo universal leucodepletion; this was introduced in 1999 to meet the concerns regarding the transmissibility of vCJD. An additional benefit of pre-storage leucodepletion may be an improvement in red cell quality since contaminating WBCs have been shown to have deleterious effects on RBCs during storage.^{138;139}

The primary function of blood is transport through flow. The primary function of RBCs is oxygen transport. RBCs must take up oxygen in the lungs, squeeze through very narrow capillaries and release the oxygen to the tissues. The loading/unloading of oxygen by haemoglobin is described by the oxygen haemoglobin equilibration curve (OHEC) see Figure 4-1. The ability of RBCs to squeeze through capillaries is a function of the red cells' deformability. A study investigating the effects of refrigerated storage on RBC deformability is reported in Chapter 5.

4.2.1 Aim

To assess the quality of the current RBC product, namely leucodepleted red cells stored in SAGM additive solution, focusing on in-vitro assays of red cell oxygenation/de-oxygenation.

4.3 The red cell storage lesion and RBC metabolism/oxygen carriage.

4.3.1 RBC physiology.

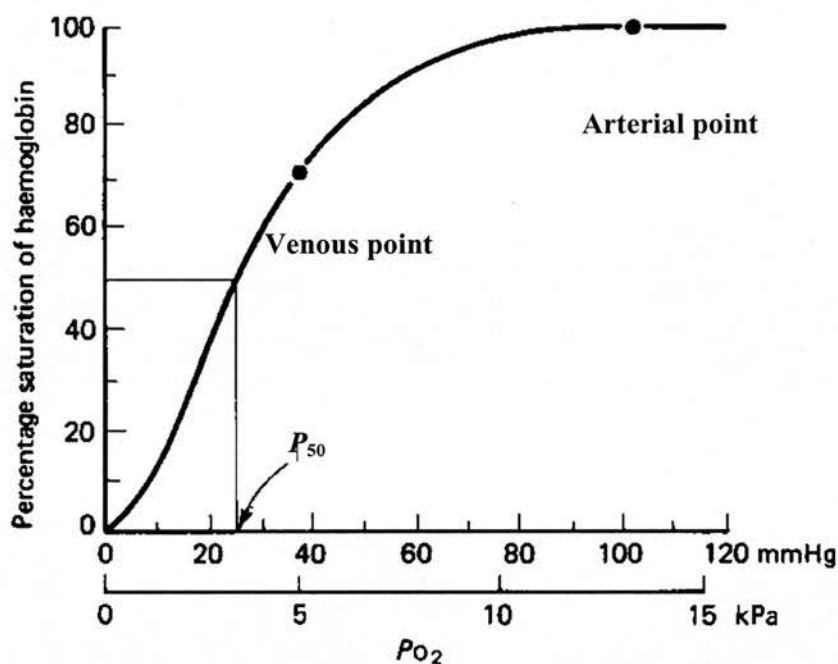
The RBC has been described as the simplest cell in the human body. Formed as a nucleated cell in the bone marrow the RBC normally loses its nucleus before being released into the circulation. On entering the circulation the RBC still possesses residual ribosomes, mitochondria and a Golgi apparatus but it normally loses these after a day or so. It then assumes its recognisable biconcave disc shape. The RBCs sole known physiological function is respiratory gas exchange. Oxygen transport is primarily by combination with Hb. Hb is found in an extraordinarily high concentration 320 g/L, in RBCs. Within the RBC Hb can transport oxygen without exerting the large osmotic effect it would have as a plasma protein. Moreover, within the RBC, Hb is protected by metabolic processes to keep the haem iron in its oxygen carrying ferrous form.

4.3.2 Oxygen carriage.

HbA₁, the commonest form of adult Hb, is a tetramer of 2 alpha and 2 beta globin chains. The spatial relationship of the 4 subunits is termed the quaternary structure of Hb. Each globin subunit has a haem group that can bind one molecule of oxygen. When a molecule of oxygen combines with the haem group of one of the subunits it alters the quaternary structure and increases the affinity of the neighbouring subunits for oxygen thereby facilitating oxygen loading. It is this facilitation of oxygenation that accounts for the sigmoid shape of the OHEC, see Figure 4-1. In comparison, myoglobin, which is a monomer and can therefore only bind one molecule of oxygen, has a oxygen equilibration curve that is a rectangular hyperbola. The shape of the OHEC is assumed to stay the same (Severinghaus 1958, 1964, 1966; Astrup 1965; Kelman 1966 cited in R Gabel Respiration Phys 1980; 42:211-32) but its position can be shifted to the left or the right. The location of the OHEC is

best described by the P_{50} . The P_{50} is the PO_2 at which the Hb is 50% saturated with oxygen, and it is used to summarize the position of the OHEC. The normal P_{50} is 3.6 kPa.

Figure 4-1 The oxygen haemoglobin equilibration curve (OHEC) showing the arterial and venous points and the P_{50} (see text for details).



Several factors can influence the position of the OHEC (Table 4-1). Hydrogen ions, carbon dioxide and 2,3 DPG all combine with Hb and decrease the affinity of Hb for oxygen. The effect of hydrogen ions on oxygen affinity is termed the Bohr effect and it is the result of the presence in Hb of amino acids, which are weaker acids in de-oxygenated Hb than oxygenated Hb. Carbon dioxide affects oxygen affinity by its influence on pH and by forming carbamino compounds.

Table 4-1 Causes of displacement of the oxygen haemoglobin equilibration curve (OHEC).

Left shift (decreased P_{50})	Right shift (increased P_{50})
Decrease in hydrogen ion concentration	Increase in hydrogen ion concentration
Decrease in temperature	Increase in temperature
Decrease in $p\text{CO}_2$	Increase in $p\text{CO}_2$
Decrease in 2,3 DPG in RBCs	Increase in 2,3 DPG in RBCs
Decrease in ATP in RBCs	Increase in ATP in RBCs
Abnormal Hbs (e.g. COHb, MetHb, fetal Hb)	Abnormal Hbs (e.g. HbS)
	Decrease in zinc in RBCs

Key:

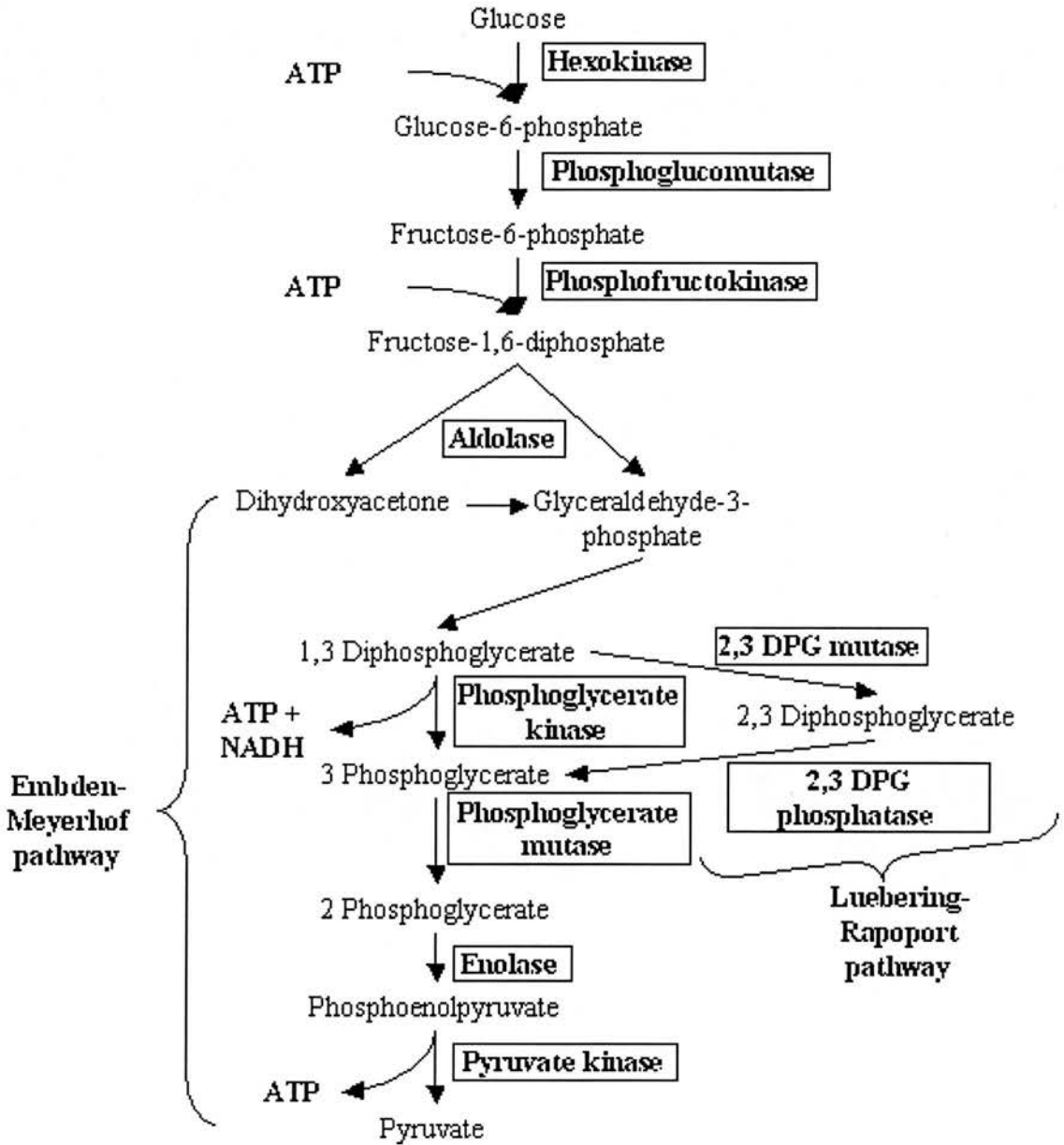
COHb Carboxyhaemoglobin

MetHb Methaemoglobin

2,3 DPG has a significant effect on the affinity of Hb for oxygen. An increase in the intra-erythrocytic 2,3 DPG concentration reduces the affinity of Hb for oxygen by two mechanisms. Firstly, 2,3 DPG binds to de-oxygenated Hb within the central cavity and stabilises it in this form. Oxygenated Hb, with its different quaternary structure, has a smaller central cavity that cannot bind 2,3 DPG. Secondly, 2,3 DPG is a strongly negatively charged polyanion. The accumulation of 2,3 DPG within the RBC lowers the internal pH and mediates a right shift of the OHEC. In fact this second mechanism may be more significant than the first.

The intra-erythrocytic concentration of 2,3 DPG is determined by its rate of synthesis and breakdown. 2,3 DPG is a product of the phosphoglycerate cycle of Rapoport and Luebering, a side shuttle of the main Embden-Meyerhof pathway, see Figure 4-2. Approximately 80%

Figure 4-2 RBC metabolism. Glucose catabolism proceeds via cleavage to trioses and then to pyruvate; the pathway through the trioses to pyruvate is called the Embden-Meyerhof pathway. 2,3 DPG metabolism is via the Luebering-Rapoport pathway.



of the glycolytic flux is via this shuttle, which means that 2,3 DPG is very plentiful in RBCs.

Factors controlling the concentration of 2,3 DPG are:

- 1) The concentration of 2,3 DPG itself. A negative feedback mechanism inhibits the activity of 2,3 DPG phosphatase.
- 2) Inorganic phosphate concentration. The 2,3 DPG concentration is low in hypophosphataemia and high in hyperphosphataemia.
- 3) The hydrogen ion concentration. Phosphofructokinase is pH sensitive and appears to be the pacemaker of the Embden-Meyerhof pathway. In alkalosis the rate of glycolysis is increased, and the conversion of glyceraldehydes-3-phosphate to 1,2 diphosphoglycerate is favoured resulting in an increased concentration of 2,3 DPG. Decreased RBC pH inhibits phosphofructokinase, slows the rate of glycolysis and activates 2,3 DPG phosphatase, which accelerates 2,3 DPG catabolism.

Other factors that affect the 2,3 DPG concentration are shown in Table 4-2.

Table 4-2. Factors influencing the 2,3 DPG concentration in erythrocytes.

Decrease in 2,3 DPG	Increase in 2,3 DPG
Increased H ⁺ concentration	Decreased H ⁺ concentration
Hypophosphataemia	Hyperphosphataemia
Hyperthyroidism	Hypothyroidism
Hexokinase deficiency	Pyruvate kinase deficiency
Old erythrocytes	Young erythrocytes
Blood storage	Anaemia

4.3.3 Estimation of P_{50} .

The P_{50} can be determined by a number of methods. Obviously, the pH and temperature at which the measurements are performed has an important bearing upon the actual value obtained.

- 1) The P_{50} can be determined by constructing the OHEC. This is done by simultaneously measuring the partial pressure of oxygen and the oxygen saturation of a blood sample during oxygenation or de-oxygenation. The measurements are usually performed at pH 7.4 and 37°C. This P_{50} value is termed the in vitro P_{50} because it is measured under in vitro conditions.
- 2) In patients with disturbances of acid-base balance and or temperature the actual in vivo P_{50} will be significantly different from the in vitro value. The P_{50} can be measured under in vivo conditions using special devices based on desaturation tonometry (such as the Van Slyke apparatus). These methods are laborious and time consuming but very accurate. A simpler approach is to estimate the in vivo P_{50} from a single point analysis of the partial pressure of oxygen and the oxygen saturation, usually from an arterial blood sample. An OHEC is then fitted to this single point and the in vivo P_{50} is estimated by extrapolation. There are a variety of algorithms available for the estimation of P_{50} many of which are incorporated into bench-top blood gas analysers.^{140;141} The in vivo P_{50} can be reported as the standard P_{50} , corrected to pH 7.4 and a temperature of 37°C, or the actual P_{50} under the actual conditions in vivo. These methods are obviously limited by the accuracy of the modelled OHEC to the actual OHEC of the blood sample being tested. These algorithms are particularly erroneous when the blood sample has a high (>92%) oxygen saturation.¹⁴²

Being a relatively simple cell the anucleate RBC has fewer metabolic needs than most other cells but it does require energy to maintain the shape and flexibility of the cell membrane, to maintain Hb iron in its functional divalent form and to preserve the intracellular ionic milieu. The RBC gets its energy from the breakdown of glucose to pyruvate via the glycolytic pathway (Figure 4-2). Glycolysis requires 2 molecules of ATP but 4 are generated resulting in a net gain of 2 molecules of ATP per molecule of glucose. In addition glycolysis provides the NADH needed to maintain the Hb in the reduced state.

4.4 Materials and methods.

4.4.1 Subjects.

The study was approved by the local research ethics committee. Ten healthy volunteers were recruited from donors attending the Scottish National Blood Transfusion Service (SNBTS) donor centre (Lauriston Place, Edinburgh, UK). All the donors met UK blood donor criteria.¹⁴³ Informed consent was obtained.

4.4.2 Blood donation and processing.

The donations were collected and processed as per SNBTS standard operating procedures. Briefly, blood was collected into quadruple packs with an integral in-line leucofilter (Figure 4-3). The primary pack contains 63 mL of citrate-phosphate-dextrose (CPD) anticoagulant. The volume of whole blood collected from each donor was $450 \text{ mL} \pm 10\%$ (Figure 4-4). The packs were cooled to and held at room temperature for 8 ± 2 hours before processing (Figure 4-5); this is to allow the donor's WBCs time to phagocytose any bacteria that may have been introduced during collection. The packs were leucodepleted using the in-line blood filters (Figure 4-6). The packs were then centrifuged at 4200 rpm for 20 minutes at $24 \text{ }^\circ\text{C}$. RBCs were separated from the plasma (Figure 4-7) and transferred to the pack containing the additive solution, SAGM. This pack was then separated by heat sealing and stored at 4 ± 2

°C for 42 days. These red cell concentrates (RCCs) represent the current red cell product in the UK.

Figure 4-3 Quadruple pack with an integral in-line leucofilter.

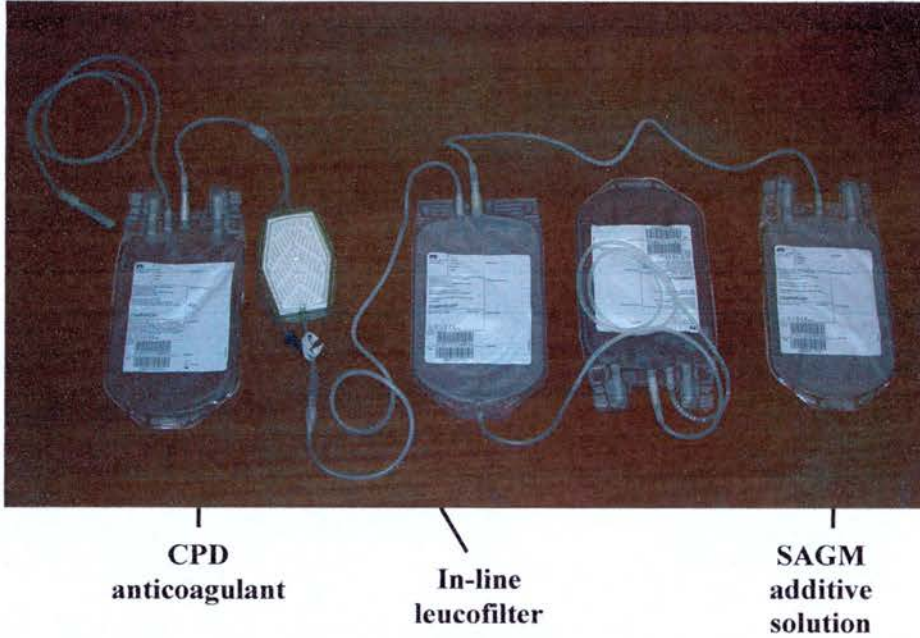


Figure 4-4 The volume of a donation is determined by weight, which equates to 450 mL \pm 10%. Because donors have different haemoglobin concentrations the donated packs can contain substantially different amounts of total haemoglobin.



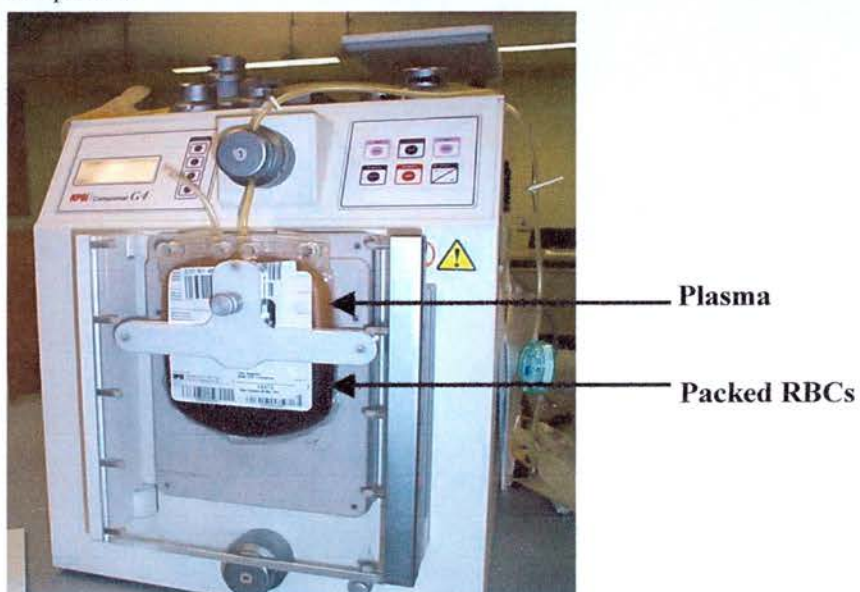
Figure 4-5 Refrigerated cooling trays are used to cool the blood donations to 20°C.



Figure 4-6 Leucofiltration. The blood donations are filtered through the in-line leucofilters under gravity. The procedure takes approximately 20-30 minutes.



Figure 4-7 The Compomat. Following centrifugation the unit is squeezed to separate out the red cells and plasma.



4.4.3 In-vitro tests.

Each RCC pack was sampled on days 0 (immediately after collection), 2 (post-processing), 7, 14, 21, 28, 35 and 42. The units were mixed thoroughly prior to sampling using a rotating blood pack mixer (Biotest Co) at 6 rpm at 4°C. Aliquots of approximately 10 mL were taken through a sampling site coupler under sterile conditions.

The following parameters were performed on all of the RCC samples:

- a) Full Blood Count (Sysmex KX21 counter, Sysmex Corporation)
- b) Blood gas analysis. Blood gas analysis was performed on days 2 and 35. A 2-point calibration was performed prior to analysis of the test samples.
- c) Plasma Hb (Kit number 527, Sigma-Aldrich Company Ltd, Gillingham, UK). The colorimetric determination of plasma Hb is based upon the catalytic action of Hb on the oxidation of tetramethylbenzidine by hydrogen peroxide. The resulting rate of colour change is proportional to the Hb concentration. The rate of colour change is detected by spectrophotometry. The exact reaction mechanism has not yet been clearly established. The following formula was used to calculate the percentage haemolysis:

Equation 4-1

$$\text{Haemolysis (\%)} = \text{plasma Hb (g/L)} \times (100 - \text{Hct}) / \text{total Hb (g/L)}$$

Reaction mixtures were prepared in test tubes to ensure adequate mixing before transferring to cuvettes. A blank and standard were prepared for each series of tests.

	Blank	Test sample	Standard
TMB solution	2 mL	2 mL	2 mL
Sample		0.01 mL	
Hb standard (30 mg/dL)			0.01 mL

At timed intervals 2 mL hydrogen peroxide solution was added to each tube. The tubes were mixed by gentle inversion. The mixture was transferred to a cuvette and left to stand at room temperature. Exactly 10 minutes after addition of the hydrogen peroxide solution the absorbance of the samples was read at 600 nm using distilled water as a reference.

The Hb concentration was calculated using the equation:

Equation 4-2

$$\text{Hb (mg/dL)} = (\text{Test} - \text{Blank}) / (\text{Standard} - \text{Blank}) \times 30$$

To determine the coefficient of variation of this assay all of the measurements of plasma Hb on samples from RCC bags 1 & 2 were performed in duplicate.

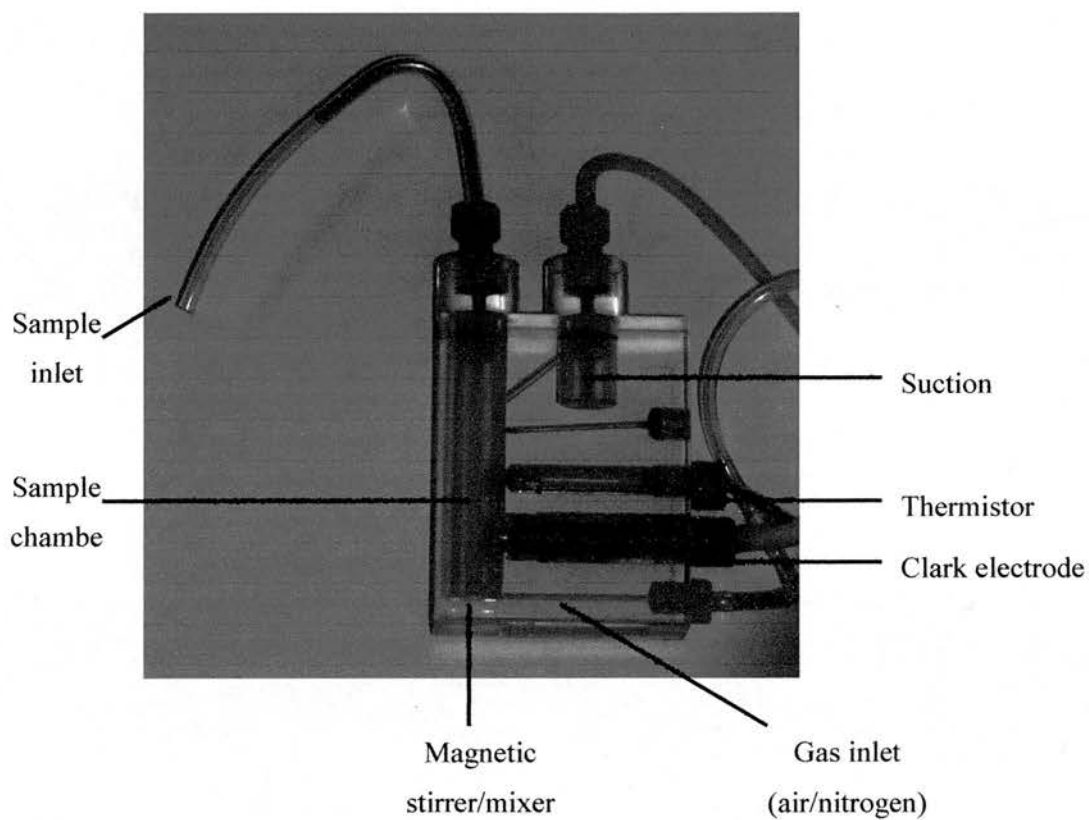
- d) Adenosine Triphosphate (ATP) concentration (Kit number 366-A, Sigma-Aldrich). The principle of this technique is described briefly here, a fuller description can be found in Section 10.1 Appendix 10.1. The technique measures the decrease in absorbance at 340 nm that results when the reduced form of nicotinamide adenine dinucleotide (NADH) is oxidized (to NAD). The amount of ATP is the rate-limiting factor. By determining the decrease in absorption the amount of ATP originally present can be determined.

- e) 2,3 DPG concentration (Kit number 35-UV, Sigma-Aldrich). The principle of this technique is described briefly here, a fuller description can be found in Section 10.1 Appendix 10.1. 2,3 DPG is hydrolyzed to 3 phosphoglycerate, which then reacts with ATP to form 1,3 diphosphoglycerate. 1,3 DPG then oxidizes NADH to NAD. Measuring the decrease in absorbance at 340 nm caused by the oxidation of NADH to NAD reflects the amount of 2,3 DPG originally present.
- f) In vitro P_{50} was measured using the Hemox Model B analyser (TCS Medical Products, Huntington Valley, Pennsylvania). This device plots the complete OHEC of a blood sample by simultaneously measuring the partial pressure of oxygen and the oxygen saturation during oxygenation or deoxygenation. The partial pressure of oxygen is measured using a Clark electrode. The partial pressure of oxygen in the blood sample can be varied by bubbling air (oxygenation) or nitrogen (deoxygenation) through the sample chamber (Figure 4-8). The oxygen saturation is measured by spectrophotometry. Fifty microlitres of blood was added to Hemox buffer pH 7.4 (also containing Additive A and anti-foaming agent). The measurements were performed at 37°C. The sample was fully oxygenated by flushing with air and then the measurements were made during deoxygenation.

4.4.4 Statistics.

The results are presented as mean (SD) unless stated otherwise. Linear regression (SPSS version 11.5.0) was used to examine the relationship between 2,3 DPG and P_{50} .

Figure 4-8 The sample chamber of the Hemox Model B Analyser



4.5 Results

Six male and 4 female donors were recruited (Table 4-3). Their mean age was 33 years (range 20 – 54 years).

Table 4-3 RCC pack and donor details

Pack	Pack volume (ml)	Total Hb (g)	Donor details		
			Blood group	Sex	Age (years)
1	264	47.3	A-	M	20
2	291	57.6	O+	M	29
3	251	47.4	AB+	F	32
4	284	57.1	B+	M	51
5	280	53.5	AB+	M	41
6	253	45.0	B+	F	20
7	277	56.0	O+	M	54
8	265	50.1	A+	M	33
9	253	46.0	O+	F	23
10	255	45.9	A+	F	28

4.5.1 RCC packs.

The RCCs had a mean volume of 267 (SD 15) mL; the minimum volume was 251 mL (Table 4-3). After leucofiltration the mean total Hb per RCC was 50.6 (SD 5) g/unit; the lowest value was 45.0 g/unit. The results of the in vitro tests before and after collection, and during 42 days of refrigerated storage are shown in Table 4-4.

Table 4-4 Assay results of the 10 donors (day 0) and their RCC donations during 42 days of refrigerated storage.

In vitro assay	Donor (Day 0)	RCC									
		Day 2	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42			
FBC	138 (11)	189 (9)	193 (16)	188 (9)	188 (10)	188 (9)	187 (9)	187 (10)			
data.											
Hb (g/L)	41.1 (2.8)	59.0 (2.6)	59.6 (3.8)	58.4(2.2)	59.4 (1.9)	59.4 (2.2)	59.7 (2.4)	59.9 (2.7)			
Hct (%)	93.7 (3.1)	98.4 (4.2)	98.7 (3.3)	98.1 (3.3)	99.4 (3.9)	99.7 (3.8)	100.2 (3.9)	101.1(4)			
MCV (fL)	31.4 (1.5)	31.5 (1.5)	31.9 (1.9)	31.5 (1.3)	31.5 (1.4)	31.5 (1.4)	31.3 (1.3)	31.7 (1.3)			
MCH (pg)	33.5 (1.0)	32.0 (1.0)	32.3 (1.3)	32.2 (1.0)	31.7 (1.1)	31.6 (1.0)	31.2 (8.6)	31.3 (.8)			
MCHC (g/L)	NA	0.06 (.04)	0.09 (.04)	0.07 (.01)	0.11 (.03)	0.12 (.03)	0.11 (.5)	0.17 (.05)			
Haemolysis (%)	14.1 (1.7)	6.9 (3.02)	3.5 (2.3)	0.7 (.7)	Undetectable						
2,3 DPG (µmol/g Hb)	27.5 (1.5)	22.65 (2.9)	20.1 (2.0)	16.9 (1.4)	17.3 (.7)	16.5 (1.0)	16.33 (.6)	15.7 (.6)			
P₅₀ (mmHg)	4.57 (.6)	4.62 (.7)	4.68 (.4)	4.56 (.4)	3.93 (.3)	3.62 (.4)	3.28 (.4)	2.82 (.4)			
ATP (µmol/g Hb)	NA	7.01 (.03)	NA	NA	NA	NA	6.44 (.06)	NA			
pH											

NA Not available.

4.5.2 FBC data

The MCV increased with increasing duration of storage, the MCH remained constant and therefore the MCHC decreased (Table 4-4).

4.5.3 Haemolysis.

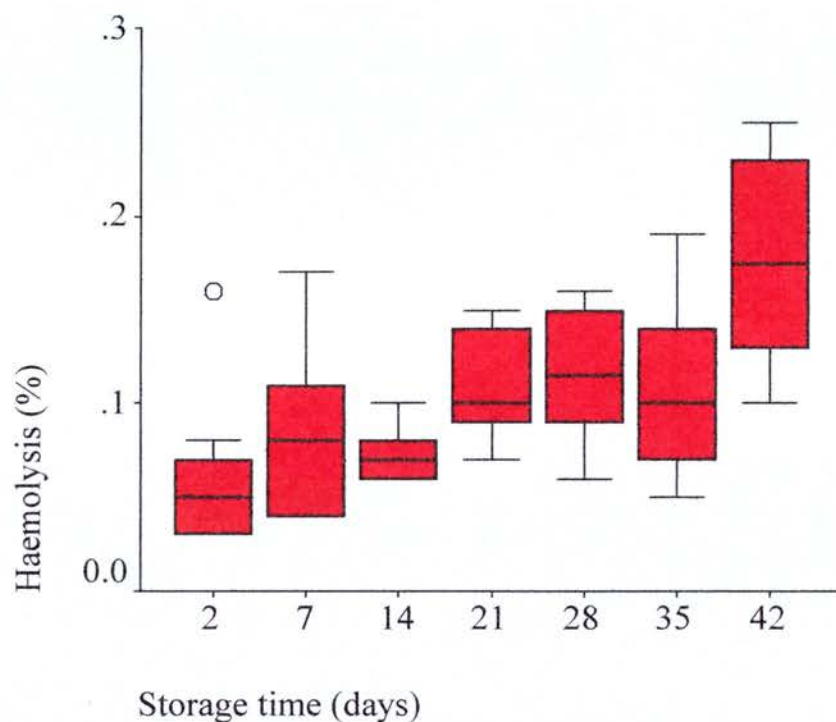
The results of the serial measurements of plasma Hb performed, in duplicate, on RCC bags 1 & 2, are shown in Table 4-5. The coefficient of variation of the plasma Hb assay was 6.6%.

Table 4-5. Serial measurements of plasma Hb performed, in duplicate, on RCC bags 1 & 2.

RCC bag	Storage age (days)	Plasma Hb (mg/dL)	
1	0	68.83	66.68
1	7	25.92	26.84
1	14	28.17	26.46
1	21	28.83	28.74
1	28	53.62	50.43
1	35	24.18	22.47
1	42	43.15	45.53
2	0	24.68	23.32
2	7	34.18	33.16
2	14	48.29	43.66
2	21	70.33	74.82
2	28	40.58	44.32
2	35	44.04	46.92
2	42	114.79	102.09

The mean percentage haemolysis increased from 0.06 (SD 0.04)% on day 2 to 0.11 (SD 0.05)% on day 35 (Figure 4-9).

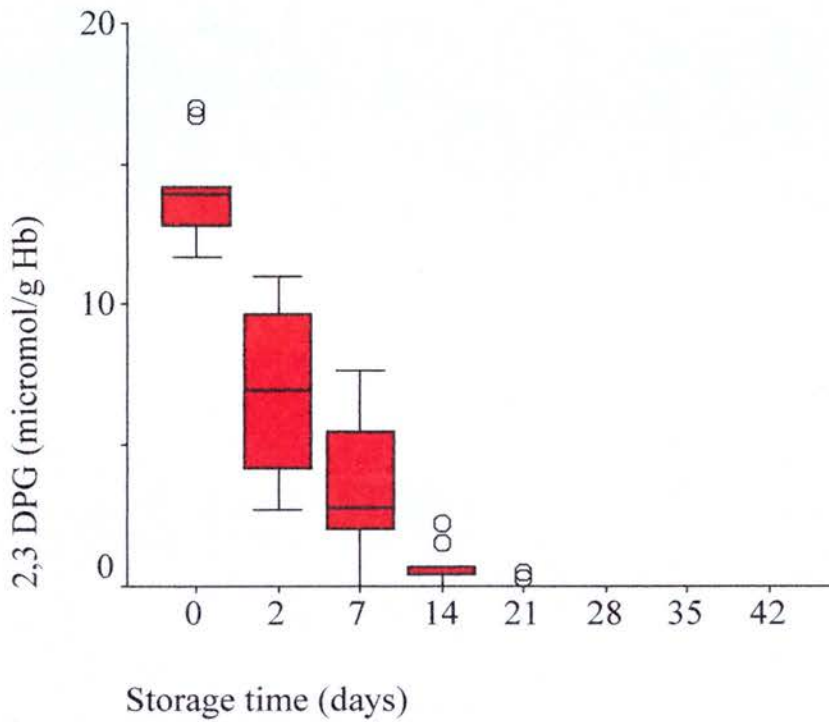
Figure 4-9 The percentage of RBC haemolysis (determined from the measurement of plasma Hb) during refrigerated storage. The hollow circle depicts an outlier (a case with a value between 1.5 and 3 box lengths from the upper or lower edge of the box).



4.5.4 2,3 DPG concentration.

The serial 2,3 DPG concentration measurements are shown in Figure 4-10. The mean 2,3 DPG concentration of the donors at the time of donation was 14.0 (SD 1.7) $\mu\text{mol/g Hb}$. On day 2, after processing, the mean 2,3 DPG concentration had decreased to 6.9 (SD 3.0) $\mu\text{mol/g Hb}$. By day 14 the mean 2,3 DPG concentration was barely detectable at 0.64 (SD 0.72) $\mu\text{mol/g Hb}$. 2,3 DPG was undetectable beyond 21 days.

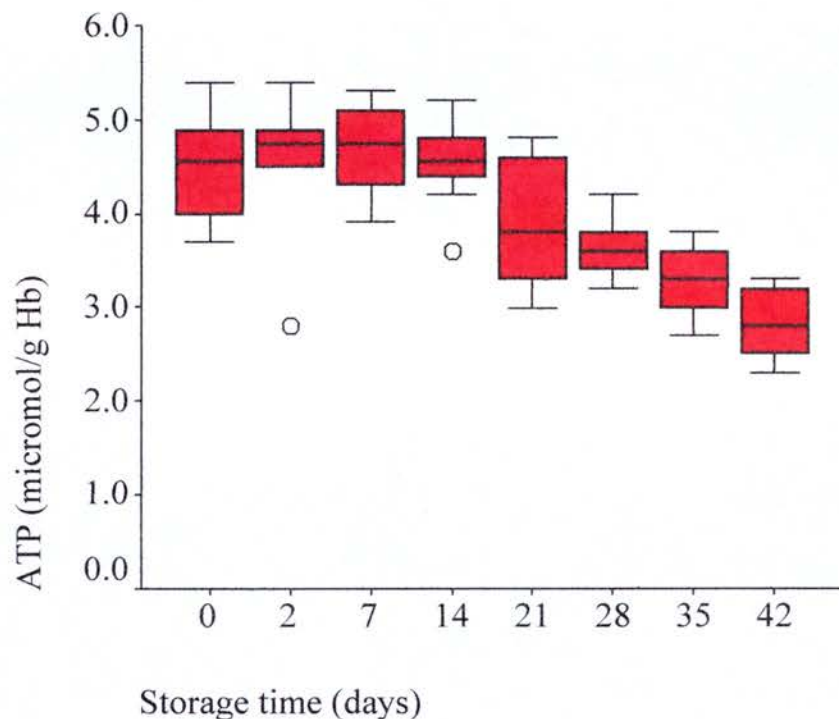
Figure 4-10 Change in RBC 2,3 DPG concentration during refrigerated storage. The hollow circle depict outliers (cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box).



4.5.5 ATP concentration.

The ATP concentration of the RCC over 42 days of refrigerated storage is shown in Figure 4-11. The mean ATP concentration in the donors on the day of donation was 4.6 (SD 0.7) $\mu\text{mol/g Hb}$. This value was maintained until day 14 after which it gradually declined to 3.3 (SD 0.4) $\mu\text{mol/g Hb}$ by day 35.

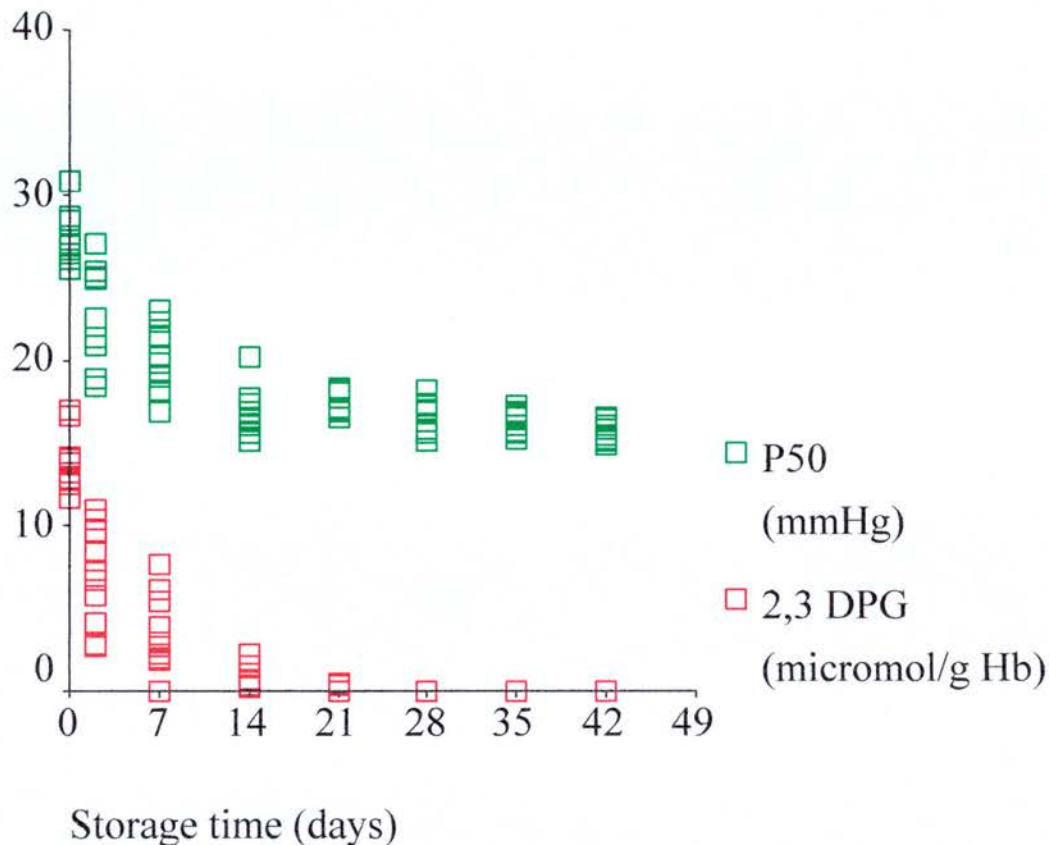
Figure 4-11 Change in RBC adenosine triphosphate (ATP) concentration during refrigerated storage. The hollow circles depict outliers (cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box).



4.5.6 In vitro P_{50} .

The mean P_{50} of the donors' blood before it had entered the collection pack was 27.5 mmHg (SD 1.5). By day 2 the in vitro P_{50} had fallen to 22.7 mmHg (SD 2.92) and by day 14 it was 16.9 (SD 1.4) mmHg. It remained relatively unchanged thereafter. The time frame for the decrease in P_{50} matched that for the decrease in the 2,3 DPG concentration (Figure 4-12).

Figure 4-12 Change in red cell P_{50} and 2,3 DPG during refrigerated storage. The P_{50} decreased during the first two weeks of storage leveled off thereafter.

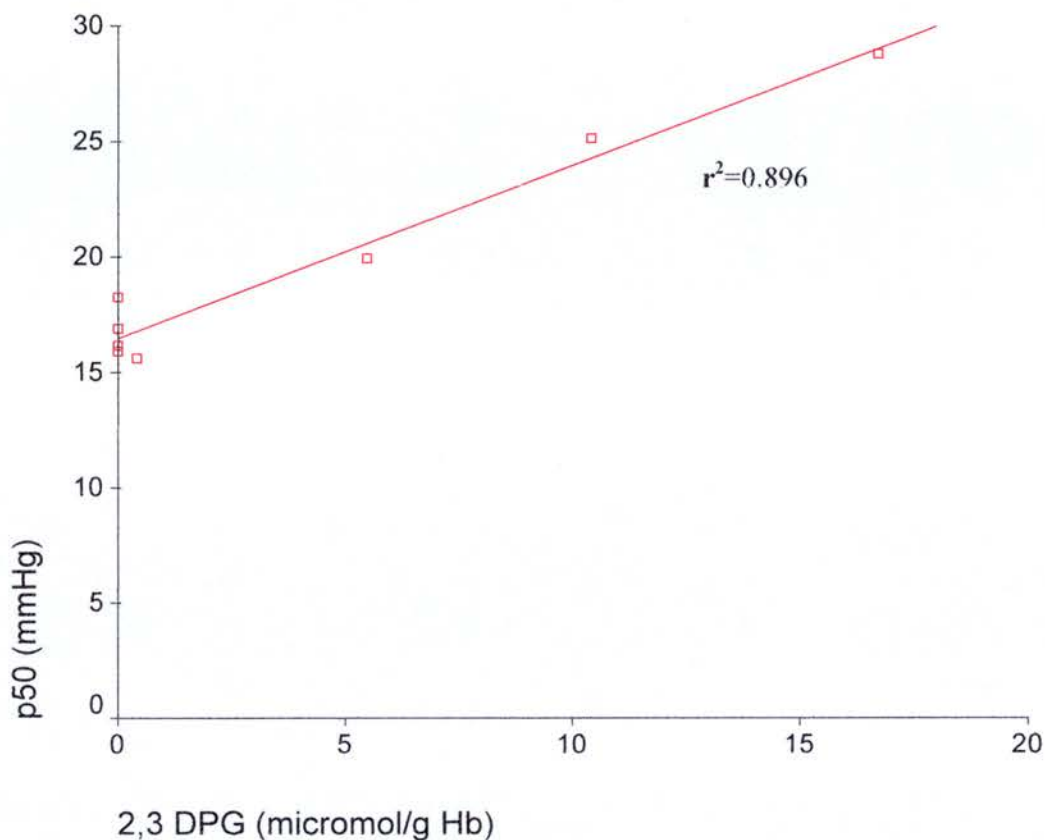


There was a strong association between the in vitro P_{50} and the RBC 2,3 DPG concentration (Figure 4-13). The mean linear regression equation was:

$$P_{50} \text{ (mmHg)} = 0.8 \times 2,3 \text{ DPG concentration } (\mu\text{mol /g Hb}) + 16.67$$

The mean coefficient of determination (r^2) was 0.91 (SD 0.035).

Figure 4-13 Scatter diagram showing the relationship between the RBC 2,3 DPG concentration and the P_{50} from one RCC (donation number 7) stored for 42 days.



4.6 Discussion.

This study has documented some of the metabolic consequences of the blood collection and storage methods currently used in the UK. Recommended tests for the evaluation of new red cell components for transfusion can be found in the “Guidelines for the Blood Transfusion Services in the UK.” (see Section 10.2 Appendix 10.2) This study chose to focus on in-vitro assays of oxygenation/deoxygenation and RBC deformability.

All the packs in this study contained >40 g of Hb, which is a recommendation under UK¹⁴³ and European guidelines.¹⁴⁴

Based on the calculated MCV, the RBCs increased in size during refrigerated storage. This is a well-recognised change and is thought to be due to the hypotonicity of the suspension medium and due to the loss of intracellular potassium by the RBCs.

There was very little haemolysis during storage. These values compare very favourably with those quoted in the literature.^{106;145}

ATP is thought to be important in maintaining RBC integrity. Studies have found associations between red cell ATP concentration and RBC deformability,¹²⁶ and the ATP concentration and post-transfusion recovery,¹²⁵ although it is far from proven that these are causal associations. In this study RBC ATP was reasonably well maintained during 35 days of refrigerated storage. Whether or not the ATP concentrations found in this study are adequate to maintain RBC integrity during storage is not known. Certainly one study found that the storage temperature, not the ATP concentration, was the rate-limiting factor for the activity of the RBC's Na^+/K^+ ATPase during refrigerated storage.¹⁴⁶ This suggests that energy depletion may not be the cause of some of the storage related changes, and attempts to improve ATP concentrations during storage may not necessarily lead to an improvement in RBC quality.

With current collection processing and storage methods RBCs lose 2,3 DPG very rapidly. Approximately 50% of 2,3 DPG had been lost by day 2 and it was barely detectable by day 14. This is similar to previous studies performed prior to the introduction of leucodepletion.¹⁴⁵ The rapid loss of 2,3 DPG may be due to the acidic pH of the anticoagulant and additive solutions (see Table 2-1). Acidosis reduces the 2,3 DPG concentration in two ways; firstly, by inhibiting phosphofructokinase and thereby reducing production of glycolytic precursors of 2,3 DPG; and secondly by simultaneously enhancing 2,3 DPG phosphatase activity causing accelerated degradation of 2,3 DPG.^{49;147;148} There are additive solutions that have improved preservation of RBC 2,3 DPG.^{106;149} Such solutions are

thought to work because they are more alkaline.¹⁵⁰ These solutions have not gained widespread acceptance because of the lack of evidence of a direct clinical benefit. The clinical importance of RBC 2,3 DPG is unclear (see Chapter 7).

This study found a very strong association between the in vitro P_{50} and the 2,3 DPG concentration in stored refrigerated blood, which is consistent with an earlier report.¹⁵¹ The earlier report used 117 pairs of 2,3 DPG/ P_{50} estimations that had been obtained from a multitude of studies; some of the studies had been investigating compounds that elevated RBC 2,3 DPG whereas other studies were investigating the decline in RBC 2,3 DPG with storage. The regression equation was:

$$P_{50} \text{ (mmHg)} = 0.667 \times 2,3 \text{ DPG concentration } (\mu\text{mol /g Hb}) + 15.61$$

The coefficient of determination (r^2) was 0.815. This compares with a mean r^2 of 0.91 (95% CI of the mean 0.869 to 0.953) for the present study. This suggests that virtually all (91%) of the variability in the in vitro P_{50} estimation was accounted for by the variability in the 2,3 DPG concentration. There appears to be very little extra information to be gained from measuring both the in vitro P_{50} and the 2,3 DPG concentration of blood during refrigerated storage.

5 The red cell storage lesion and red blood cell deformability.

5.1 Abstract.

RBCs must be able to squeeze through very narrow capillaries in order to deliver the oxygen to tissues. The ability of RBCs to squeeze through narrow capillaries is a function of the cell's deformability. The quality of the current red cell product, namely leucodepleted RBCs stored in saline-adenine-glucose-mannitol additive solution, was assessed using an assay of RBC deformability, the St George's filtrometer. A before-and-after-storage study design was used. The filterability of RBCs from 10 time-expired (storage time 35 days) red cell concentrates and 10 healthy volunteers were compared. The results were expressed as the standard instrument parameter red cell transit time (RCTT) in seconds. The mean (SD) RCTT of the fresh and stored RBCs was 9.97 (0.89) seconds and 12.57 (1.84) seconds respectively.

Current blood collection, processing and storage methods result in a slight reduction in RBC filterability. Whilst this observed decrease in RBC filterability was statistically significant ($p=0.032$), its clinical significance is not known. Filterability assays assess global RBC deformability and are unable to identify the cause of the decrease in deformability.

5.2 Introduction.

Blood rheology is the science of the flow and deformation of blood and blood cells. The primary function of blood is transport by flow. Rheology is important because the major components of resistance to blood flow are vascular and the “thickness” or “stickiness” of blood itself i.e. its viscosity. Viscosity is due to the internal friction between adjacent layers of a liquid. In laminar flow in tubes adjacent layers of liquid move parallel to each other, but faster in the centre than at the sides. The velocity gradient between any two planes in flow is known as the shear rate and the force required to produce this gradient is called the shear stress. The viscosity of a fluid is the shear stress divided by the shear rate.

5.3 Determinants of blood viscosity.

The factors that determine blood viscosity can be classified into 3 broad categories: plasma properties, RBC properties and the RBC concentration.

5.3.1 Plasma properties.

The increase in plasma viscosity above the viscosity of water is almost entirely due to the plasma proteins. This is only relevant in certain disease states, such as multiple myeloma or Waldenstrom’s macroglobulinaemia where there is excessive production of a monoclonal immunoglobulin.

5.3.2 RBC properties.

The RBC properties that influence blood viscosity can be thought of as properties of RBCs in suspension and properties of an individual cell. There is a natural tendency for RBCs in suspension to repel each other. But at low shear rates and in the presence of macromolecules, such as acute phase proteins, this electrostatic repulsion may be overcome causing the RBCs to aggregate. The haematocrit also has an important effect on RBC aggregation, with a peak effect at around 40-45%.

The deformability of individual RBCs can also influence blood viscosity. The deformability of the RBC is determined by its geometry, the viscosity of the intracellular contents and the viscoelastic properties of the cell membrane; viscoelasticity is the term used to describe the behaviour of some materials that have the properties of both a solid (elasticity) and a liquid (viscosity).

RBC geometry.

The size, shape and surface area/volume ratio of the RBC affect its deformability. The larger the cell the greater the degree of deformation required for it to pass through a restrictive aperture. It has proved difficult to attribute the effect of cell shape on deformability mainly because of the difficulties of quantifying cell shape abnormalities. The relationship between cell surface area and volume determines the extent to which a cell can deform. A spherical cell cannot change its shape unless it increases its area or decreases its volume. A normal red blood cell has a 50% excess surface area compared to a sphere of the same volume.

Intracellular viscosity.

The viscosity of a solution of haemoglobin increases exponentially as its concentration rises above 300 g/L. The mean cell haemoglobin concentration (MCHC) is approximately 330 g/L. A direct relationship between MCHC and viscosity has been demonstrated¹⁵² although other factors such as the physico-chemical state of the Hb are also important.¹⁵³

Membrane properties.

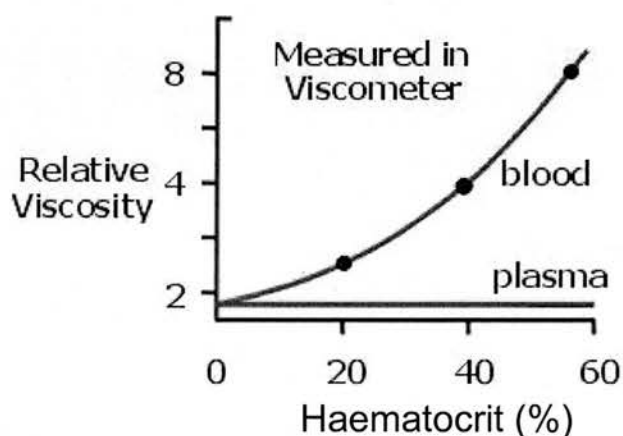
In the course of RBC deformation its membrane might expand, shear or bend. The RBC membrane is highly resistant to expansion. RBC deformation therefore takes place by a combination of shearing and bending. To enter very narrow vessels RBCs must elongate and shearing will dominate, whereas bending may dominate in larger vessels. The membrane is viscoelastic; it has viscosity, a resistance to flow, and elasticity, a capacity to return to its

original form. The viscous and elastic components depend on the protein cytoskeleton underlying the membrane bilayer.

5.3.3 RBC concentration.

The addition of RBCs to plasma disturbs flow streamlines and progressively increases viscosity. Newtonian fluids, such as plasma, have a constant viscosity, whereas at low shear rates the viscosity of whole blood increases as the haematocrit increases (Figure 5-1). This is primarily due to RBC aggregation to form rouleaux (stacks of ≥ 3 RBCs). In fact, haematocrit is the most important single determinant of whole blood viscosity. In polycythaemia, especially polycythaemia rubra vera, the incidence of vaso-occlusive episodes increases markedly when the haematocrit increases above 60%.

Figure 5-1 The relationship between haematocrit and blood viscosity (published with kind permission from Dr Richard Klabunde, <http://www.cvphysiology.com>)



In addition the haematocrit varies at different sites in the circulation due to plasma skimming. It can therefore be seen that the resistance of blood to flow, its viscosity, will be different at different points in the circulation.

From the foregoing discussion it is apparent that the transfusion of stored blood may affect the recipient's blood viscosity by:

- 1) Introducing a population of RBCs that are affected by storage (and may be less deformable).
- 2) Increasing the haematocrit. In clinical practice this effect will be slight since most patients are transfused only 1 or 2 units of RBCs at a time, which produces only a modest increase in the haematocrit, and therefore the increase in blood viscosity would be expected to be small.

Since RBCs are plasma depleted blood transfusion should have very little effect on plasma viscosity.

5.4 Measurement of RBC deformability.

Early studies used osmotic fragility as an assay of RBC deformability.¹⁵⁴ Nowadays there are more specific and sensitive assays for the assessment of RBC deformability. Rheological methods can be divided into those that test bulk suspensions of RBCs and those that test individual cells. Measures of deformability are dependent on the technique used and it is not possible to compare the results of the different techniques.

5.4.1 Shear methods.

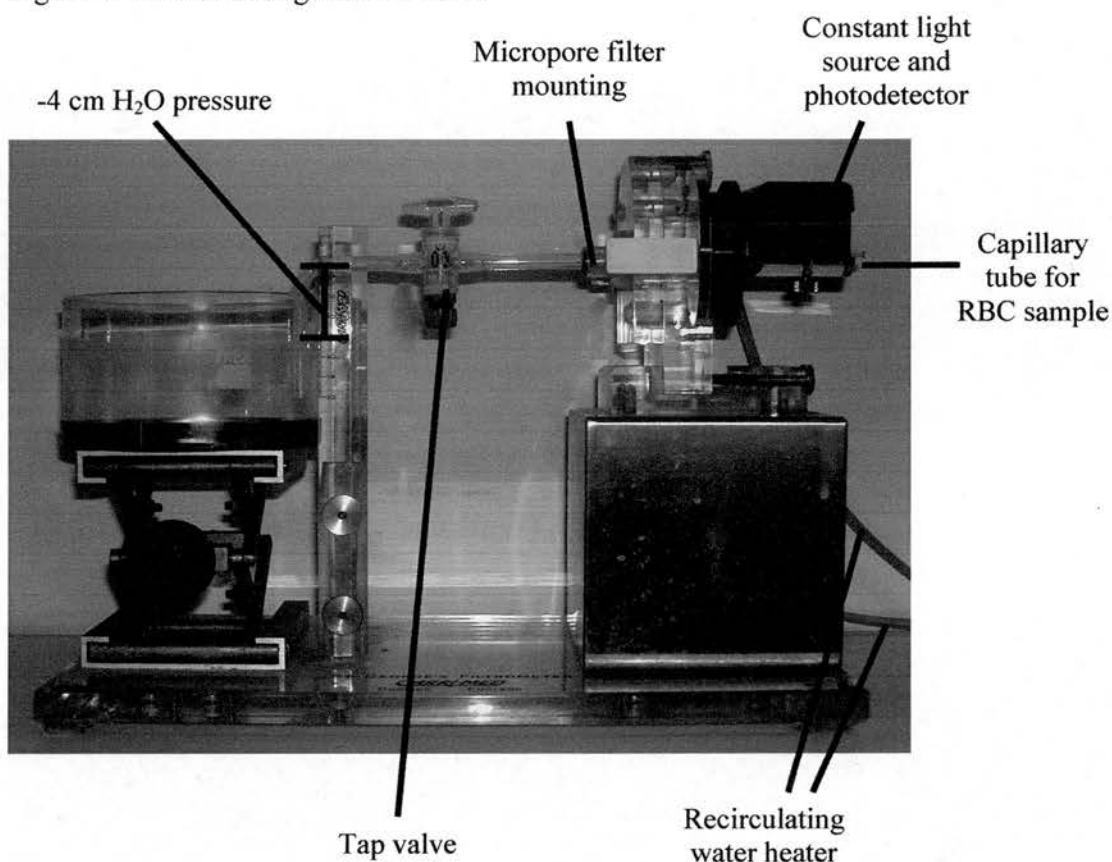
When whole blood or a RBC suspension in buffer is spun in a rotational viscometer (rheometer) any RBC aggregates are dispersed and the RBCs are deformed into elliptocytes whose membranes move in tank tread-like motion around the cytoplasm. The ektacytometer combines a rheometer with a laser that allows the ellipticity of the RBCs to be measured on the laser diffraction image.

5.4.2 Filtration methods.

Filtration of RBCs through polycarbonate membranes has been the most widely used method for measuring deformability. Commercial filtrometers are usually of the initial flow rate design where only a small volume of RBC suspension is filtered. The St George's filtrometer (Figure 5-2) was one of the more widely used filtrometers. The St George filtrometer consists of a glass capillary tube with a constant light source on one side and a photodetector on the opposite side. The capillary tube is mounted upstream from a polycarbonate membrane; the polycarbonate membrane contains pores of 5 μm diameter and 10–12 μm length. The capillary tube is first primed with a fixed volume of buffer solution. On turning a tap the buffer solution is drawn across the filter by a negative hydrostatic pressure. The time taken for the fixed volume of buffer to pass through the filter is accurately recorded by the photodetector. The process is then repeated with the RBC test suspension using the same membrane. The membrane is changed after each RBC test suspension. The rate of pore transit is expressed as a ratio of the flow rate of the RBC suspension in buffer to that of the buffer alone. For the St George's filtrometer this ratio is expressed as the red cell transit time (RCTT) in seconds. The membranes can be cleaned ultrasonically and re-used thereby improving precision and avoiding the variation that occurs between batches of membranes (variations in pore density and/or pore diameter).¹⁵⁵ Particular attention must be paid to the preparation of the test sample since contaminating white blood cells have a dominant effect on filterability.¹⁵⁶

As a rule filtrometers test bulk suspensions not individual cells. The Cell Transit Analyser is the exception to this rule, it can measure electronically the passage of individual RBCs using a computer system. It is able to achieve this because it uses membranes with only 30 pores.

Figure 5-2 The St. George's filtrometer.



5.4.3 Micropipette techniques.

The deformability of individual cells can be tested by measuring the time taken, or the pressure required, for entry into glass micropipettes with an internal diameter of 3-3.5 μm . Narrower pipettes can be used to test the properties of the membrane specifically rather than the cell as a whole.

5.5 RBC deformability in disease.

From the foregoing discussion it is apparent that RBC deformability is dependent upon many different factors. Therefore abnormalities of RBC deformability may result from many causes. Indeed abnormalities of RBC deformability have been described in a wide variety of disease states such as various haemolytic anaemias, sickle cell anaemia, malaria, diabetes mellitus, renal failure and sepsis.^{23;24;157-159} Studies in animal models have found that less

deformable RBCs have a detrimental effect on regional blood flow.¹⁶⁰⁻¹⁶² It has also been suggested that the microcirculatory changes associated with sepsis may be a consequence of altered RBC rheology.¹⁶³ Although abnormal RBC deformability may result in tissue ischaemia, such as in sickle cell anaemia, or premature RBC destruction, such as spherocytosis or elliptocytosis, the clinical significance of abnormalities of RBC deformability in the majority of disease states is not known.

5.6 RBC deformability and blood storage.

Several studies have investigated the effects of blood storage on RBC deformability (Table 5-1).

During storage there is a loss of cellular potassium accompanied by cell swelling and a loss of membrane lipid vesicles (approximately 25% of membrane phospholipid is lost during 42 days of storage)¹¹¹ These changes are thought to have a deleterious effect on RBC deformability.¹⁶⁴

Most, but not all, of the studies that have investigated the effects of refrigerated storage on RBC deformability have found that deformability decreases with increasing duration of storage (Table 5-1). However it is difficult to draw any firm conclusions from these studies because there were major methodological differences between them. For instance, different RBC products were studied; various combinations of whole blood, packed RBCs, buffy coat reduced RBCs, storage solutions and storage times were included. Notably, none of the studies looked at leucodepleted RBCs. In addition, different assays of deformability have been used and even where studies have used the same assay there were important methodological differences. For example, of those studies that used filtration techniques, some, but not all, performed a WBC reduction procedure, such as buffy coat removal or filtration through Imugard cotton wool, on the test RBC suspension. Also, different studies used different filters; it has already been shown that there can be significant differences

between different batches of the same type of filter.¹⁵⁵ Furthermore, even when studies have used similar assays the results may have been presented in formats that make comparisons impossible. Finally, 5 out of the 9 cited studies analysed ≤ 5 RBC units.

One of the earliest studies investigating the effects of blood storage on RBC deformability (using an ekacytometer) found that there was no gross loss of deformability until the RBCs had been stored long enough for the ATP levels to have decreased to less than 30% of initial values.¹²⁶ However, when the measurements were performed in hypotonic media, which increases the sensitivity of the method, subtle alterations in RBC deformability were detectable even though ATP concentrations were not reduced.

Another early study found that the storage of whole blood in ACD resulted in very early and marked changes in RBC deformability.¹²⁷ This study used a filtration technique and leucodepleted the samples using Imugard cotton wool just prior to testing. After 7 days of storage filterability had decreased to 19 & 14% of baseline. Notably, blood from only 2 subjects was tested.

More recent studies have used the ektacytometer to measure RBC deformability. In one study,¹⁶⁵ RBC deformability decreased by approximately 10% after 7 days and by 50-60% after 35 days of storage in SAGM. Again it was a small study (n=3). The other study, whose main aim was to investigate the effects of x-ray irradiation on RBCs prior to routine blood storage, found a progressive reduction in RBC deformability during storage in the non-irradiated controls (n=5).¹⁶⁶ Irradiation was found to have a detrimental effect on RBC deformability in addition to the effect of storage.

Table 5-1. Summary of the studies that have investigated the change in RBC deformability during refrigerated blood storage.

Reference.	RBC product.	Deformability assay.	Results.
127	WB in ACD anticoagulant (n=2). Storage time ≤20 days	Filtrometer (Myrenne type). WBC reduction using Imugard cotton wool. Mynipore Nickel 7 µm filter	30% decrease in the filtration index after 11 days, 42% after 20 days.
167	CPD anticoagulant. Packed RBCs in SAGM (n=10). Buffy coat depleted. Storage time 35 day	Filtrometer (Custom made). No WBC removal performed. Cellulose filter.	No significant change in filterability.
168	WB in CPD anticoagulant (n=12). Storage time 14-26 days (median 18.5 days)	Filtrometer (Custom made). No WBC removal performed. Nucleopore 5 µm filter.	Transit time of fresh blood 1.3 mseconds and stored blood 3.65 mseconds.
169	WB in CPD anticoagulant (n=5). Storage time 35 day	Filtrometer. Buffy coat removal. Cellulose filter.	Marginal, but not significant (5%) increase in the filtration time (time taken for fixed volume of RBCs to pass through a filter).
170	Packed RBCs in adenine saline solution (n=9). Storage time 42 days	Filtrometer (custom built). Buffy coat removal. Nucleopore 5 µm filter.	Deformability index (DI) significantly reduced (118.9 to 70.9) by 14 days of storage.

Reference.	RBC product.	Deformability assay.	Results.
126	WB in CPD (n=9) and packed RBCs in CPD (n=10). Storage time 42 days.	Ektacytometer.	Progressive decrease in DI with increasing storage and increased packing (increased Hct) of RBCs during storage. Change in DI became significant after 28 and 35 days for packed RBCs and WB respectively.
166	ACD anticoagulant. Packed RBCs in mannitol adenine phosphate solution (n=5). Storage time 28 days	Ektacytometer (LORCA).	DI decreased from a mean (SD) of 0.35 (0.019) on day 2 to 0.278 (0.028) by day 28 ($p < 0.02$).
165	CPD anticoagulant. Packed RBCs in SAGM (n=3). Storage time 40 days	Ektacytometer (LORCA).	Decrease in RBC elongation index starting in first week and progressing throughout duration of storage (decreasing from 0.57 to 0.25, approximately).
114	Packed RBCs in CPDA-1 solution (n=5). Storage time 29 days	Micropipette technique.	34% decrease in membrane deformability after 4 weeks.

None of these studies looked at leucodepleted RCCs. As mentioned earlier, leucodepletion could potentially improve the quality of stored RBCs.^{138;139} Leucodepletion could potentially improve the preservation of RBC deformability during storage.

5.6.1 Aim

To compare the deformability of “fresh” and stored (leucodepleted RCCs) RBCs. Deformability was assessed using the St George’s filtrometer. The rationale for the use of the St George’s filtrometer is shown in Table 5-2.

Table 5-2 Rationale for the use of St George's filtrometer.

Advantages:

- i. Until relatively recently, filtration techniques were the most used method for the assessment of RBC deformability. The St George's filtrometer has been used by a number of investigators.
- ii. They test a bulk suspension of RBCs and provide data on a large representative number of cells.
- iii. Filtration is a global assessment of RBC deformability.
- iv. Filtration assays are relatively simple and cheap.

Disadvantages:

- i. The St George's filtrometer is no longer commercially available. In addition it requires an obsolete BBC computer to run the software.
- ii. Meticulous preparation of the test sample is required because residual WBCs can occlude the filter pores.¹⁵⁶
- iii. Filtrometers do not test cell subpopulations.
- iv. Filtrometers are unable to give any clue as to the underlying structural basis of a defect in RBC deformability.

5.7 Materials and methods.

5.7.1 Test RBC suspensions.

Suspensions of “fresh” and stored RBCs were prepared. A power analysis, using a well-known nomogram,¹⁷¹ estimated that a comparison of 10 “fresh” RBC suspensions with 10 stored RBC suspensions would be able to detect a red cell transit time (RCTT) difference of 2.5 seconds, assuming a SD of 2 seconds, a power of 0.8 and a significance level of 0.05. A RCTT difference of 2.5 seconds was thought to be the smallest difference that could reliably be detected.

The “fresh” RBCs were obtained from 11 healthy volunteers (HVs). One HV was bled on 10 separate occasions and the other 10 HVs were bled on 2 separate occasions. Each sample consisted of 10 mL venous blood collected into EDTA anticoagulant.

The stored RBCs were obtained from 10 time-expired (35 days) RCC packs that were provided by SNBTS. The donations had been collected and processed as per SNBTS standard operating procedures (see section 4.4.2). A 10 mL sample was taken from each pack after thorough mixing using a rotating blood pack mixer (Biotest Co.) at 6 rpm. The age and sex of the blood donors was obtained from computerised records in the SNBTS Donor Centre (Lauriston Place, Edinburgh). No other donor details were recorded.

5.7.2 RBC suspension preparation.

Leucocyte removal was performed on all the blood samples using the Imugard IG 500 cotton wool (Terumo Corporation, Tokyo, Japan) pre-filtration technique.¹⁷² HEPES (hydroxyethyl piperazineethanesulfonic acid) buffer (Sigma-Aldrich, Gillingham, UK) was used throughout (pH 7.4, osmolality 290 mmol/kg). The samples were then washed twice in HEPES buffer. The RBCs were then suspended in HEPES buffer to a haematocrit of 0.07, which was checked using a full blood count analyser (Sysmex KX21 counter).

5.7.3 Filtration.

A St. George's filtrometer (Carri-Med Ltd., Dorking, UK) with temperature control was used with a driving pressure of -4 cm H₂O. Filtration was measured at 37 °C using the same batch of polycarbonate membranes of 5 µm pore diameter (Nucleopore Corporation, Pleasanton, California). After use the membranes were cleaned ultrasonically and re-used.¹⁵⁵ The results were expressed as the standard instrument parameter red cell transit time (RCTT) in seconds. Each sample was tested in triplicate and the expressed results are the mean of these triplicates.

5.7.4 Data analysis

The results are presented as mean (SD) unless stated otherwise. Because repeated measurements of RCTT (on the same sample, or on the same HV on two or more occasions) were performed we were able to determine the within-sample and within-subject coefficients of variation (CV) as practical indices of measurement error. The distribution of the RCTT data was examined for normality using Q-Q probability plots. The results of the first sample obtained from each of the 10 HVs were compared with the results of the stored RBCs. The comparison was made using both Student's independent t test and the Mann Whitney U test.

5.8 Results

The age and sex of the HVs and of the blood donors are shown in Table 5-3.

Table 5-3 Age and sex of the healthy volunteers and blood donors.

	Healthy volunteer (n=1)	Healthy volunteers (n=10)	Stored RBCs (n=10)
Age	36	47 years	38 years
Mean			
(Range)		(37 – 60)	(20 – 48)
Sex (M:F)	M	5:5	5:5

The red cell transit time (RCTT) results are shown in Figure 5-3 and Table 5-4. There was no evidence that the RCTT data was not normally distributed. Therefore the RCTT data comparisons were made using Student's independent t-test and the Mann Whitney U test.

Figure 5-3 Box plots of the red cell transit time (RCTT), in seconds, for RBCs from 10 healthy volunteers (10 HV) and from red cell concentrates stored for 35 days (stored). Also shown (as a measure of reproducibility) are the filterability results obtained from one healthy volunteer (1 HV) performed on ten separate occasions. The hollow circle depicts an outlier (a case with a value between 1.5 and 3 box lengths from the upper or lower edge of the box).

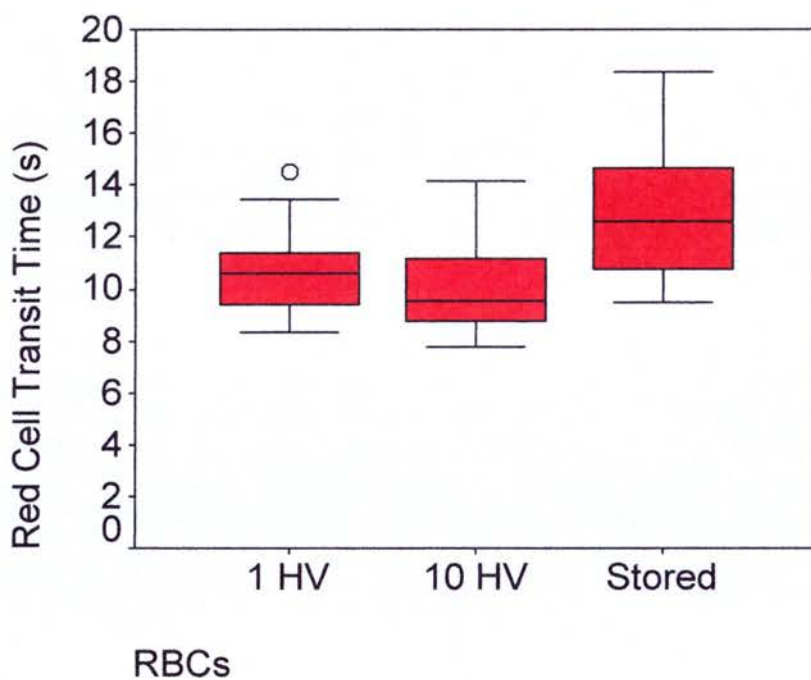


Table 5-4 Full blood count parameters and red cell transit times (RCTTs) of the RBC suspensions. The results are presented as mean and SD. The within subject and within sample coefficients of variation (CV) of the RCTT are also presented.

		Healthy volunteers		Stored RBCs
		(n=1)	(n=10)	(n=10)
MCV (fL)	Mean	95.5	91.1	86.8
	SD	0.82	3.8	6.2
MCH (g/L)	Mean	33.1	30.6	30.5
	SD	1.16	1.74	3.34
MCHC (pg/L)	Mean	346	340	355
	SD	10	9.3	21
RCTT (seconds)	Mean	10.61	9.97 *	12.57 *
	SD	0.91	0.89	1.84
95% CI of the mean RCTT		10.03 to 11.19	9.41 to 10.53	11.41 to 13.73
within subject CV (%)		8.56	9.92	
within sample CV (%)		13.55	14.11	13.59

Key

* p=0.032 (Student's independent t-test) and p=0.02 (Mann Whitney U test)

5.9 Discussion.

Current SNBTS blood collection, processing and storage methods result in a small but statistically significant loss of RBC deformability after 35 days of refrigerated storage.

This study was a “before-and-after” comparison study; “fresh” RBCs from HVs were compared with stored RBCs from time-expired leucodepleted RCCs. This approach was adopted in order to maximise the observed difference.

The two groups, the blood donors and the HVs, were comparable; they had the same male to female ratios and all of the subjects met SNBTS donor criteria. The mean age of the HVs was slightly higher than the mean age of the blood donors.

The “fresh” RBCs from the 10 HVs had a mean RCTT of 9.97 (0.89) seconds. This is slightly lower than the normal value quoted in the literature (11.0 (0.8) seconds) for this technique.¹⁷³ The results of this study were reproducible and consistent. The within-sample CVs for the single HV, the 10 HVs and the stored RBCs was 13.55, 14.11 and 13.59% respectively, although these values are higher than the value of 7.7% reported in the literature.¹⁷³ The within-subject CVs were also similar, 8.6% for the individual HV and 9.9% for the 10 HVs; again these values were slightly higher than the value of 5.1% reported in the literature.¹⁷³ These inter-laboratory differences maybe due to differences in the membranes or buffer. The choice of buffer can certainly have a significant effect on the reproducibility of the filterability assay (see Section 10.3 Appendix 3).

This study found that stored RBCs had a statistically significant reduction in filterability compared with “fresh” RBCs. The cause of this difference in RBC filterability is unclear. Unfortunately, filterability assays assess global RBC deformability and are unable to elucidate the cause. Refrigerated storage may have a detrimental effect on RBC deformability via a variety of different mechanisms. RBC geometry is an important determinant of RBC deformability and a high percentage of RBCs lose their biconcave shape and become echinocytic or spherocytic during refrigerated storage.¹⁰⁹ This shape change is initially reversible but with increasing duration of storage becomes irreversible.¹⁷⁴ Stored RBCs taken straight out of storage have an increased MCV (Table 4–4); this is thought to be

caused by a net efflux of potassium, which is a result of the decreased activity of membrane channels and pumps due to refrigeration.¹⁴⁶ The increased MCV is unlikely to account for the reduced deformability of stored RBCs because variations of MCV and MCHC within the normal range have been found to have little, if any, effect on the RCTT.¹⁷⁵ In actual fact, in this study, the stored RBCs had a smaller mean MCV than the fresh RBCs (91.1 and 86.8 fL respectively, see Table 5-4) and smaller than stored RBCs taken straight from the RCC bag (Table 4-4). This may simply be due to sampling error (different donors) but it may also be due to the fact that, in this study, the stored RBCs were incubated in HEPES buffer at 37°C prior to analysis, which is known to correct the intracellular potassium concentration and reverse the cell swelling.^{146;176} Other aspects of the red cell storage lesion may have a detrimental effect on RBC deformability. After approximately 2 weeks of storage RBCs shed membrane in the form of microvesicles.¹⁰⁹ These microvesicles are about 100 to 200 nm in diameter and are composed of mainly phospholipid, being relatively protein-poor compared to the normal RBC membrane. Microvesicle formation is irreversible resulting in a loss of membrane and a reduction in the surface area to volume ratio that is so fundamental to RBC deformability. RBC protein oxidation is known to occur during storage but its effects on deformability are purely speculative.^{177;178} Altered distribution of membrane phospholipids is also known to occur during refrigerated storage and this may have significant consequences for the RBC;¹²⁸ phosphatidyl serine (PS) is normally only present on the inner surface of the cell membrane but in acid pH such as with refrigerated storage it accumulates on the outer membrane where it appears to result in RBC destruction. Again the effect of this change on RBC deformability is not known.

Interestingly, a very recent study,¹⁷⁹ using an ektacytometer, found no change in the deformability of leucodepleted RBCs during 5-6 weeks of refrigerated storage. The present study found a modest reduction in the deformability of RBCs that had undergone pre-storage leucodepletion. The comparative sensitivities of the ektacytometer and the St George's

filtrimeter are not known but these findings suggest that the presence of WBCs during storage may have a detrimental effect on RBC deformability. Certainly, pre-storage leucodepletion of RBC products has been shown to decrease the accumulation of leucocyte derived cytokines and granule contents, such as IL-8 and neutrophil elastase, with increasing duration of storage. It is not inconceivable to imagine that other substances released by leucocyte or platelet degranulation, such as proteases or reactive oxygen species, could have a detrimental effect on the RBC deformability.

Although this study found a decrease in RBC deformability, which did reach statistical significance, it was only a very modest change. For comparison, RBCs from patients with sickle cell anaemia, who suffer occlusive crises due to the poor deformability of the sickled RBCs, have a very prolonged RCTT, mean (SD) 87.5 (60.4) seconds.¹⁷³ The clinical significance of a reduction in RBC deformability of the magnitude found in this study is not known. Although blood storage procedures should attempt to maintain near normal RBC physiology, there is currently insufficient evidence to advocate the use of RBC deformability assays to routinely assess the quality of RBC storage. More studies are needed to:

- Determine the sensitivity and reproducibility of the various assays of RBC deformability.
- Compare the deformability of non-leucodepleted and leucodepleted RBCs during refrigerated storage.
- Determine the clinical significance of the red cell storage lesion. This can only be answered by comparing the clinical efficacy of fresh and stored RBCs. This issue is discussed further in Chapter 9.

6 The viability of transfused red blood cells in critically ill patients.

6.1 Abstract.

The transfusion of stored blood to critically ill patients has been found to produce only a modest and transient increment in the Hb concentration. RBC survival is an important aspect of the efficacy of allogeneic blood transfusion. Radiolabel studies were performed to determine the post transfusional recovery of stored red blood cells in critically ill patients. Twenty-one non-bleeding critically ill patients who required a blood transfusion for anaemia (mean haemoglobin concentration 76 g/L, range 65 to 85 g/L) were recruited. A dual radiolabel technique was used. Allogeneic transfused red blood cells were labeled with ^{51}Cr and the patient's autologous red blood cells were labeled with $^{99\text{m}}\text{Tc}$. The radiolabelled RBCs were injected at the end of the transfusion. Serial blood samples were taken and counted in a dual channel gamma counter. Standard techniques were used to correct for overlap of spectra, background and decay. The $^{99\text{m}}\text{Tc}$ count rates were corrected for radiolabel elution using a correction factor derived from data obtained from a separate group of 8 patients. The mean storage age of the red cell concentrates was 22 days (range 10 to 29 days). The mean recovery at 24 and 48-hour was 91% and 88% respectively.

These data show that leucodepleted red cell concentrate, suspended in saline-adenine-glucose-mannitol and stored for up to 29 days, has good 24 and 48-hour recovery in critically ill patients.

6.2 Introduction.

Anaemia is a common finding in critically ill patients. Until recently allogeneic RBC transfusion was the only therapy available to most patients. Approximately 40% of critically ill patients receive a blood transfusion in the ICU,^{1;2} rising to 73-85% in patients with a prolonged ICU admission (>7 days).⁷ About half of these RBC transfusions are to treat anaemia in the absence of acute bleeding. Recent evidence suggests that restrictive transfusion triggers should be used in the critically ill.⁶⁵ Even when these are used significant numbers of ICU patients will require red cells because critical illness is associated with a high prevalence of anaemia and a progressive decrease in Hb concentration.¹⁸⁰

An audit of transfusion practice in the ICU found that while the mean increase in Hb concentration on the day after transfusion with leucodepleted RBCs was 6 g/L per unit, this had decreased to 3 g/L per unit by about 48-hours.¹⁸¹ This transient increase in the Hb concentration raised the possibility that stored allogeneic RBCs may have significantly reduced viability in critically ill patients. A number of studies have demonstrated that RBCs from critical ill patients, particularly critically ill patients with sepsis, have reduced deformability compared to RBCs from control groups.²²⁻²⁴ A separate study found an association between loss of RBC deformability and reduced RBC viability.²⁶ It has also been shown that activated phagocytes exaggerate the removal of senescent RBCs and those coated with immunoglobulins or immune complexes.^{182;183}

When red cells are stored in the liquid state, they progressively lose their ability to survive in-vivo.¹⁸⁴ By convention, the term post transfusional recovery (PTR) is used to describe the survival of RBCs at 24 hours post transfusion. The term RBC survival is used to describe RBC survival beyond 24 hours. Currently accepted standards, for RBC products, require a mean in vivo recovery of the transfused RBCs of 75% or better 24-hours after infusion.¹⁸⁵ Coupled with this is a requirement that the mean minus 2 standard errors of the mean

exceeds 70%. Most of the data concerning the 24-hour recovery of stored RBCs are from studies of autologous transfusion in healthy volunteers.¹⁸⁴ There are no data about the 24-hour recovery of stored allogeneic red cells in the critically ill.

6.2.1 Aim

To determine the 24 and 48-hour recovery of stored allogeneic RBCs following therapeutic transfusion to critically ill patients. A secondary aim was to compare RBC recovery in septic and non-septic critically ill patients.

6.3 Methods.

The study was approved by the local research ethics committee and by the UK administration of radioactive substances advisory committee (ARSAC). Informed assent was sought from the patient's next of kin.

6.3.1 Patients and setting.

The study was conducted in the ICU of a University Teaching Hospital. Ventilated critically ill patients were eligible for inclusion if they had an Hb concentration <90 g/L and the intensivist in charge of the patient had decided that they needed a RBC transfusion. Patients were excluded if they were younger than 18 years, were women of childbearing age (<50 years), had evidence of blood loss, were haemodynamically unstable or had another contraindication to RBC transfusion.

6.3.2 Study protocol.

Patients were recruited into two groups, the main study group and an elution group. The purpose of the elution group was to determine the rate of ^{99m}Tc loss from the labelled RBCs so that ^{99m}Tc count rates could be corrected for this.

6.3.3 Main study group.

Patients in the main study group had allogeneic RBCs labelled with ^{51}Cr and autologous RBCs labelled with $^{99\text{m}}\text{Tc}$.

6.3.4 Elution group.

Patients in the elution group had autologous RBCs labelled with ^{51}Cr and $^{99\text{m}}\text{Tc}$.

The clinical management of patients was dictated by the responsible ICU consultant and was not altered for the purposes of the study. Demographic data together with diagnostic and sequential organ failure assessment (SOFA) scores were recorded for all patients.¹⁸⁶ Sepsis was diagnosed by standard clinical and haematological criteria with microbiological evidence of infection.¹⁸⁷

6.3.5 Size of the study.

It was estimated that the standard deviation of the 24-hour recovery of the current UK red cell product in critically ill patients could be up to 15% (based on data in the literature).¹⁸⁴ It was calculated that 21 patients were required in the main study group to allow differentiation between a 24-hour recover of 75% and 85% with a power of 0.9. The calculation was performed using a one-sample t-test with a one-sided significance level of 5%.

A further eight patients were recruited to the elution group.

The total study population therefore consisted of 29 critically ill patients (main study group $n=21$ and elution group $n=8$).

6.3.6 Red cell units.

Each patient was crossmatched for two units of RCC. The RCCs were issued using the standard SNBTS practices. No attempt was made to influence the storage time of the RCCs although units of an equivalent storage time were chosen if possible, which is standard practice in our hospital blood bank. The RCCs were from routine blood bank stock and had

been collected, processed and stored using SNBTS standard operating procedures. Briefly, all donations were collected into citrate-phosphate-dextrose (CPD) anticoagulant, leucofiltered at the time of initial component preparation, plasma depleted and re-suspended in SAGM additive solution.

6.3.7 Blood samples taken for radiolabelling of RBCs.

The following blood samples were taken before the start of the transfusion.

Main study group.

For labelling with ^{51}Cr , a 10 mL sample was withdrawn from each RBC unit (after thorough mixing by hand).¹⁸⁸ The two samples were then mixed together in a sterile vial to provide a single sample representative of the allogeneic transfusion.

For labelling with $^{99\text{m}}\text{Tc}$, a vial of Technescan PYP (Mallinckrodt Medical B.V., Petten, Holland) was reconstituted with 6 mL 0.9% sodium chloride. Two mL of this solution was administered by direct intravenous injection. After 20 minutes 5 mL of blood was drawn from the patient via an indwelling arterial cannula. This sample was injected into a sterile vial containing 25 units Heparin.

The elution group.

For labelling with ^{51}Cr , 20 mL of the patient's autologous blood was withdrawn and injected into a vial containing 2.5 mL CPD anticoagulant.

For labelling with $^{99\text{m}}\text{Tc}$, a sample of the patient's autologous blood was obtained using the same method as for the main study group.

6.3.8 Radiolabelling.

Radiolabelling was performed in an aseptic environment (Americare compact blood labelling suite) using aseptic technique.

Labelling with ^{51}Cr was performed according to the ACD-A method recommended by the International Committee for Standardisation in Haematology (ICSH).¹⁸⁸ Briefly, the citrated blood sample was centrifuged at 300 g for 10 minutes and then the supernatant plasma was removed and discarded. Sodium Chromate [^{51}Cr] solution (2 MBq in 0.2 mL) was added to the packed RBCs. The mixture was incubated at room temperature for 15 minutes with intermittent gentle mixing. The ^{51}Cr -RBCs were washed twice in 4-5 volumes of sodium chloride 0.9% solution. The supernatant liquid was retained and its activity was later measured to allow calculation of the labelling efficiency. The ^{51}Cr -RBCs were re-suspended in sodium chloride 0.9% solution to a volume of approx 20 mL.

Labelling with $^{99\text{m}}\text{Tc}$ was performed using an in-vivo/in-vitro labelling technique.¹⁸⁹ Briefly, Sodium Pertechnetate [$^{99\text{m}}\text{Tc}$] injection (2 MBq) was added to the heparinised blood. The mixture was incubated at room temperature for 30 minutes. The $^{99\text{m}}\text{Tc}$ -RBCs were washed in sodium chloride 0.9% solution and re-suspended to a volume of approx. 12 mL.

6.3.9 Preparation of standard solutions.

A standard solution was prepared for each radionuclide by diluting a known volume (determined by weight) of radiolabelled RBCs to 100 mL with H_2O . An aliquot of the solution was pipetted into a sample tube for counting with the blood samples.

6.3.10 The blood transfusion.

Each unit of RCC was given via a blood-warming device over 1 hour.

6.3.11 Administration of labelled blood.

The $^{99\text{m}}\text{Tc}$ labelled and ^{51}Cr labelled RBCs were injected at the end of the transfusion. The mass of radiolabelled cells administered was determined by the following method. An empty syringe was weighed. The radiolabelled cells were drawn into the syringe and it was re-weighed. The contents were injected via an indwelling venous cannula. To ensure complete

emptying of the syringe it was flushed twice by withdrawing sodium chloride 0.9% solution from a flush system attached to the cannula and injecting it into the patient.

6.3.12 Sampling.

At 5, 7.5, 10, 15 minutes 1, 4, 8, 24 and 48 hours after administration of the radiolabelled RBCs 5.5 mL samples of venous blood were collected into weighed heparinised tubes. The blood samples were drawn from a site other than that used for injection. The Hb concentration was determined for each sample using a Full Blood Count analyser (Sysmex KX21 counter, Sysmex Corporation, Kobe, Japan). The tubes were reweighed and the mass of blood in each sample was calculated. From the mass, the volume of blood in each sample was calculated.

6.3.13 Sample processing.

The cells in the blood samples were lysed by the addition of saponin to provide an homogeneous sample. Each sample and standard was counted for 10 minutes in a dual channel automatic gamma counter (Compugamma, LKB Instruments). Standard techniques were used to correct the count-rate of each radionuclide for background, overlap of the gamma-ray spectra, decay during the counting session and sample volume. The activity of the sample was expressed as counts per minute (cpm) per g Hb.

6.3.14 1^o outcome:

To determine the 24-hour recovery of stored allogeneic RBCs in critically ill patients.

6.3.15 2^o outcomes:

- i. To determine the 48-hour recovery of stored allogeneic RBCs in critically ill patients.
- ii. To compare RBC recovery in septic and non-septic patients.
- iii. To document the change in the Hb concentration over the immediate 48 hours after therapeutic blood transfusion.

iv. To investigate for associations between:

A. RBC recovery and the change in Hb concentration

B. RBC recovery and the storage age of the transfused RBCs.

6.3.16 Data analysis.

The 24-hour recovery of the ^{51}Cr allogeneic RBCs was determined by both a single and dual label technique.

6.3.17 Single label technique.

This technique used the ^{51}Cr data only. RBC recovery at time t was determined using Equation 6-1:

Equation 6-1

$$\text{RBC recovery at time } t \text{ (\%)} = \frac{\text{cpm } ^{51}\text{Cr per g Hb at time } t \times 100}{\text{cpm } ^{51}\text{Cr per g Hb at time-zero}}$$

The time-zero value (which represents 100% RBC recovery) was determined by the early time values averaged method. This is simply the average of the count rates of the early (5, 7.5, 10 and 15 minutes) samples.

6.3.18 Dual label technique.

The recovery of allogeneic RBCs labelled with ^{51}Cr was compared to the recovery of the patient's own RBCs labelled with $^{99\text{m}}\text{Tc}$. Back extrapolation on the $^{99\text{m}}\text{Tc}$ counts was performed to determine the time-zero value. This value was used to estimate the patient's red cell mass and then estimate the ^{51}Cr time-zero value. The 24-hour recovery of the ^{51}Cr labelled allogeneic RBCs was calculated as a ratio of $^{99\text{m}}\text{Tc}$ and ^{51}Cr at 24 hours to the ratio of $^{99\text{m}}\text{Tc}$ and ^{51}Cr at time-zero (Equation 6-2):

Equation 6-2

$$\begin{aligned} \text{24-hour recovery} &= \frac{\text{cpm } ^{51}\text{Cr at 24 hours} \times ^{99\text{m}}\text{Tc time-zero value}}{\text{of } ^{51}\text{Cr labelled RBCs} \quad ^{51}\text{Cr time-zero value} \times \text{cpm } ^{99\text{m}}\text{Tc at 24 hours} \times \text{CF}} \end{aligned}$$

Where CF is a correction factor for $^{99\text{m}}\text{Tc}$ elution. The correction factor was calculated using data obtained from the elution group (Equation 6-3):

Equation 6-3

$$\begin{aligned} \text{Correction factor} &= \frac{\text{24-hour recovery of autologous } ^{51}\text{Cr RBCs}}{\text{24-hour recovery of autologous } ^{99\text{m}}\text{Tc RBCs}} \end{aligned}$$

In calculating the correction factor the ^{51}Cr labelled autologous RBCs were assumed to have a 24-hour recovery of 100%. No correction was made for ^{51}Cr elution over the 24-hour period. The $^{99\text{m}}\text{Tc}$ time-zero values of the elution group were calculated by back extrapolation as described above.

An analysis of the RBC survival in patients with and without a diagnosis of sepsis was performed using the Mann Whitney U test.

6.4 Results.

The clinical features of the 29 patients are shown in Table 6-1. Five patients had incomplete SOFA scores because neurological evaluation was not possible due to the use of sedation and muscle relaxants.

6.4.1 The main study group.

Fifteen patients had received a transfusion earlier during their hospital admission but no one had been transfused for 3 days prior to enrolment. The mean Hb concentration immediately prior to transfusion was 76 g/L (range 65 to 85 g/L). The mean storage age of the red cell concentrates was 22 days (range 10 to 29 days), see Figure 6-1.

Table 6-1 Patient details.

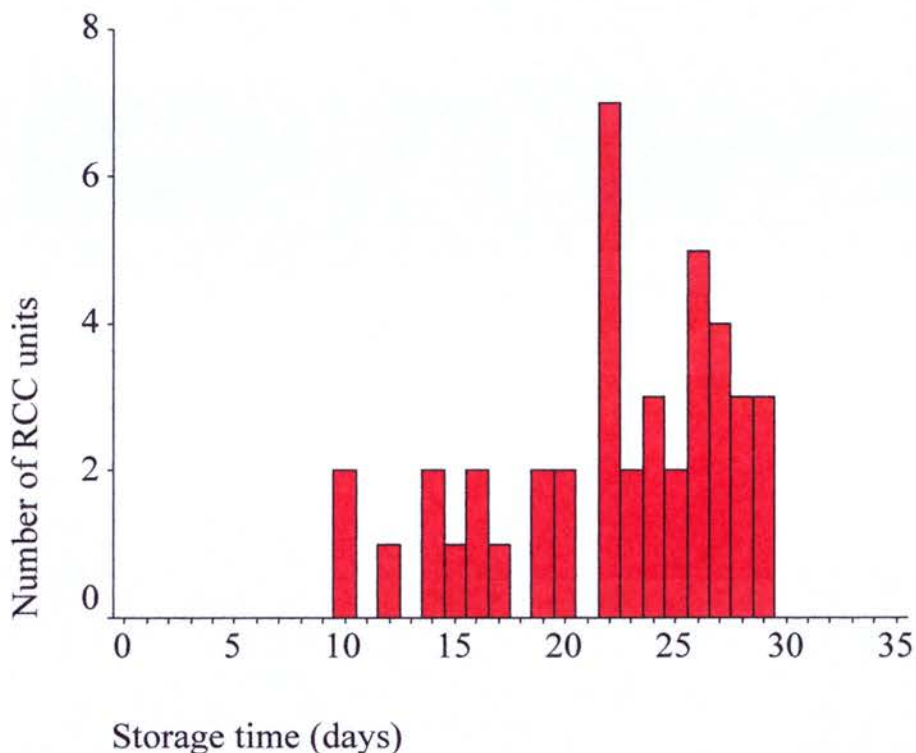
APACHE III Diagnosis	Age (years)	Sex	ICU LOS	Previous		SOFA score							
				Transfusion? (days previously)	Sepsis	Resp	Liver	CNS	Coag	CVS	Renal	Total	
1 Pneumonia-bacterial	64	M	17	N	Y	3	0	1	0	0	0	3	7
2 Pneumonia-bacterial	77	F	4	N	N	3	0	0	0	1	1	3	7
3 Trauma-multiple site w/o head/brain	57	M	20	Y(9)	Y	3	0	0	0	1	1	3	7
4 Pneumonia-bacterial	45	M	33	Y(6)	Y	3	0	0	0	0	0	3	6
5 Other respiratory disorder	76	M	3	N	Y	3	0	0	0	0	0	0	3
6 Peritonitis	50	M	10	Y(9)	Y	3	2	0	0	0	0	1	6
7 ARDS	66	M	24	Y(4)	Y	3	0	1	2	0	0	0	6
8 Pneumonia-bacterial	71	M	26	Y(7)	Y	3	0	1	0	0	0	0	4
9 Pancreatitis	61	M	15	Y(7)	N	3	1	1	1	0	0	1	6
10 GI perforation/rupture	66	F	5	Y(5)	Y	3	0	1	2	1	1	0	7
11 Septic shock-GI tract	50	F	19	N	Y	3	0	0	0	0	0	0	3
12 Aortic aneurysm rupture	68	M	20	Y(4)	N	1	2	NA	0	0	0	1	4
13 Pneumonia-bacterial	67	F	6	Y(6)	Y	3	0	3	0	3	3	3	12
14 Other renal disorder	62	F	8	Y(4)	Y	3	2	0	0	0	0	1	6
15 GI obstruction	60	M	14	Y(4)	N	2	0	0	1	3	3	3	9
16 Pneumonia-bacterial	72	M	11	Y(3)	Y	3	0	1	0	0	0	0	4

APACHE III Diagnosis	Age (years)	Sex	ICU LOS	Previous		SOFA score							
				Transfusion? (days previously)	Sepsis	Resp	Liver	CNS	Coag	CVS	Renal	Total	
17 Pancreatitis	42	M	10	N	Y	3	2	NA	0	0	0	0	5
18 Trauma-extremities	55	M	3	N	Y	3	1	NA	4	4	1	1	13
19 Pneumonia-bacterial	75	F	6	N	Y	3	0	1	0	0	0	0	4
20 Aortic aneurysm rupture	63	M	27	Y(5)	Y	3	3	1	0	4	4	4	15
21 Pneumonia-bacterial	75	M	32	Y(27)	Y	4	0	1	0	0	0	0	5
22 Pneumonia-bacterial	74	F	7	N	Y	4	0	NA	1	0	0	0	5
23 Adrenal neoplasm	77	M	19	Y(12)	Y	3	0	0	0	0	3	3	6
24 GI perforation/rupture	75	F	21	Y(22)	Y	3	0	0	0	4	0	0	7
25 Trauma-multiple site w/o head/brain	73	F	13	Y(8)	Y	3	2	0	0	0	0	0	5
26 Pneumonia-bacterial	79	M	57	Y(14)	N	3	0	1	0	0	0	0	4
27 Septic shock-GI tract	65	M	9	Y(7)	N	3	0	NA	0	4	0	0	7
28 Pneumonia-bacterial	84	M	41	Y(17)	Y	2	0	0	0	0	0	0	2
29 GI perforation/rupture	68	M	53	Y(6)	Y	3	0	0	0	0	0	0	3

Group summaries	Age		ICU LOS		Previous transfusion		Total SOFA score	
	Mean (range)	Gender M:F	Median (range)	Sepsis (n)	Mean (range)	Sepsis (n)	Mean (range)	
Main study group (n=21)	65 (42-84)	18:3	15 (3-57)	15	16	6 (2-15)		
Elution group (n=8)	68 (50-77)	2:6	17 (6-21)	6	7	6 (3-12)		

Key: APACHE III Acute physiology and chronic health evaluation III
ICU LOS Intensive care unit length of stay
SOFA score Sequential organ failure assessment score
ARDS Acute respiratory distress syndrome
NA Not available due to the use of sedation/muscle relaxants
Shaded area Elution group
w/o without

Figure 6-1 Storage time (days) of the red cell concentrates (RCC) transfused to patients in the main study group.



6.4.2 The elution group.

Six patients had received a transfusion earlier during their hospital admission but no one had been transfused for 4 days prior to enrolment. The mean Hb concentration immediately prior to transfusion was 73 g/L (range 64 to 89 g/L). The mean storage age of the red cell concentrates was 14 days (range 6 to 22 days)

6.4.3. Single-label technique.

The 24 and 48-hour recovery of allogeneic ^{51}Cr labelled RBCs following transfusion to critically ill patients is shown in Table 6-2 and Figure 6-2.

Figure 6-2 The 24 and 48-hour recovery of stored allogeneic RBCs in critically ill patients.

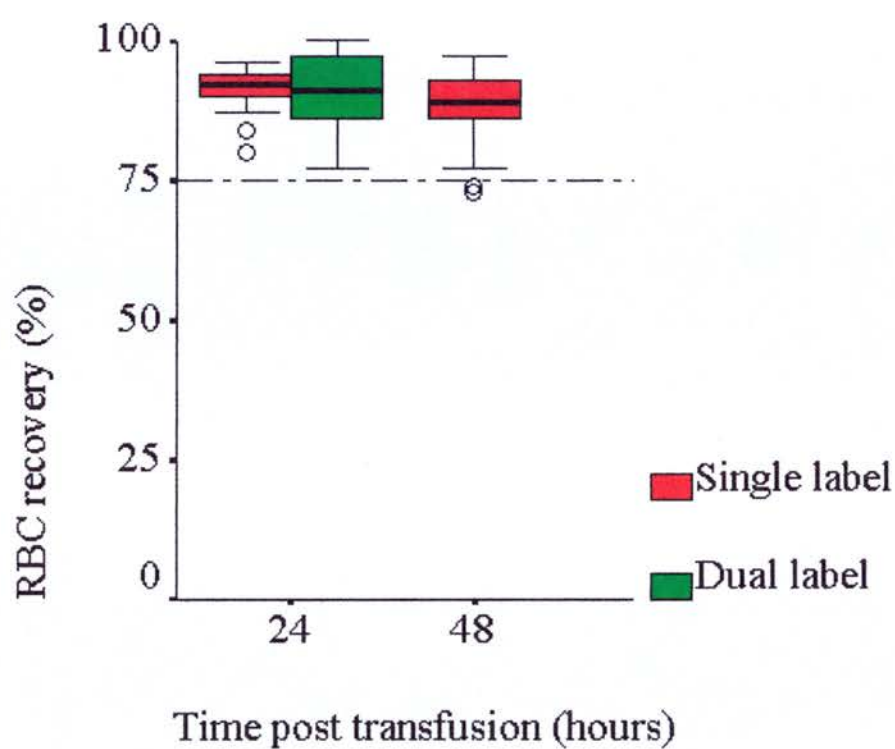


Table 6-2 The 24 and 48-hour recovery of allogeneic ^{51}Cr labelled RBCs in critically ill patients. RBC recovery was estimated by single and dual-label techniques. Recovery values are expressed as a % of the RBCs estimated to be present at time zero. An estimate of 48-hour RBC recovery was not available using the dual-label technique.

Patient ID	24-hour recovery (%)		48-hour recovery (%) Single-label	RCC storage age (days)
	Single-label	Dual-label		
1	96	91	95	26 & 26
2	94	90	93	26 & 27
3	95	91	87	15 & 16
4	95	80	97	28 & 29
5	91	100	92	14 & 14
6	96	97	97	23 & 23
7	92	91	91	26 & 26
8	93	93	87	16 & 17
9	87	86	74	10 & 24
10	80	88	73	25 & 25
15	93	97	91	10 & 12
16	93	80	86	22 & 22
17	87	77	87	24 & 27
18	92	96	89	29 & 29
19	84	79	80	27 & 27
20	94	87	93	20 & 20
21	90	99	77	22 & 22
26	90	99	89	22 & 24
27	87	86	93	28 & 28
28	91	100	88	22 & 22
29	92	100	84	19 & 19
Mean	91	91	88	22
SD	3.9	7.5	6.9	
(95% CI of mean)	89-93	88-94	85-91	

6.4.4 Dual-label technique.

The rate of ^{99m}Tc elution was high; a mean of only 71% of the label was present at 24 hours

Table 6-3. The 24-hour ^{99m}Tc elution correction factor was calculated as 1.41 (95% CI 1.31-1.51) see Equation 6-4.

Table 6-3 The 24-hour recovery of autologous RBCs in the elution group.

Patient ID	24-hour recovery of ^{99m}Tc labelled RBCs (%)
11	61
12	78
13	68
14	73
22	77
23	79
24	65
25	64
Mean (SD)	71 (7)
Median	70.5

Equation 6-4

$$\begin{aligned} \text{Correction factor} &= \frac{\text{24 hour recovery of } ^{51}\text{Cr labelled RBCs}}{\text{24-hour recovery of } ^{99m}\text{Tc labelled RBCs}} \\ &= 1.41 \end{aligned}$$

The 24-hour recovery of allogeneic RBCs determined by a dual-label technique is shown in Table 6-2 and Figure 6-2.

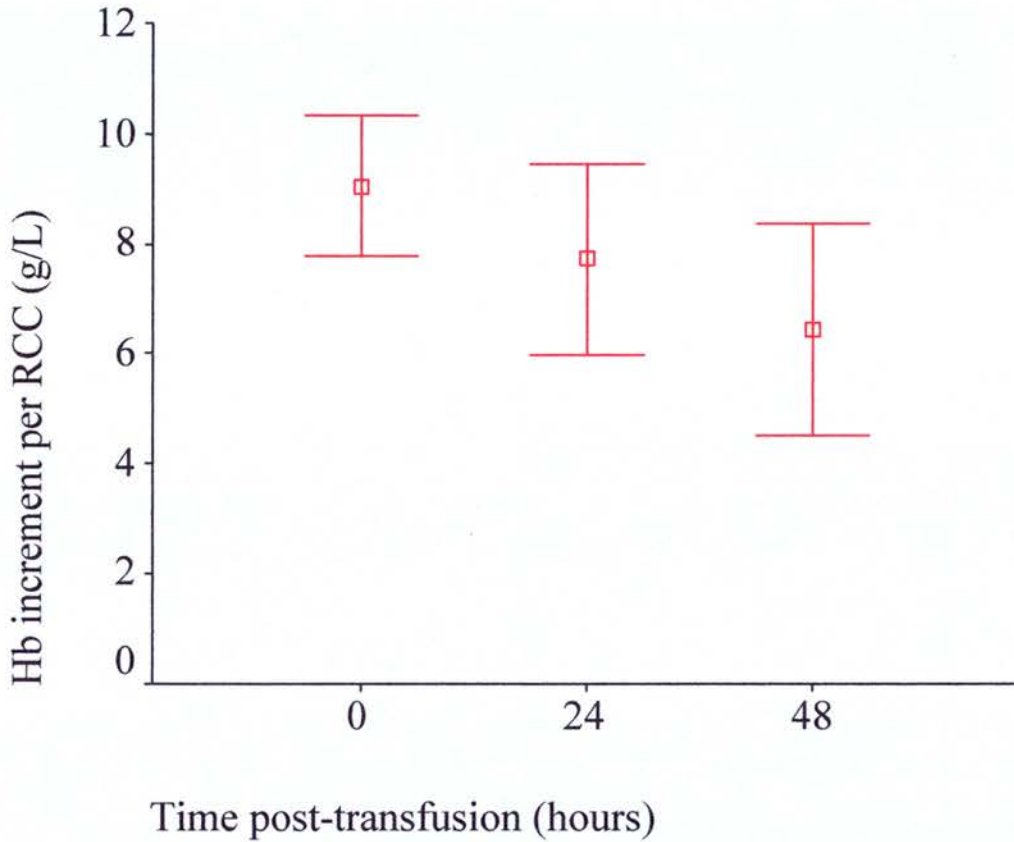
There was no significant difference in RBC recovery between septic and non-septic patients Table 6-4.

Table 6-4 RBC recovery in septic and non-septic patients. The results are presented as median and inter-quartile range (IQR). A comparison between the 2 groups was performed using the Mann-Whitney U test.

Patient group	24-hour RBC recovery		48-hour RBC recovery Median (IQR)
	Single label Median (IQR)	Dual label Median (IQR)	
Septic	92 (90 – 95)	91 (82 – 99)	88 (85 – 93)
Non-septic	90 (87 – 94)	90 (86 – 98)	91 (82 – 93)
	p = 0.41	p = 0.87	p = 0.62

The change in the Hb concentration over the first 48 hours following transfusion is shown in Figure 6-3. There was no association between the Hb increment and the 24-hour recovery determined by single and dual label techniques ($r = 0.34$ and 0.14 respectively).

Figure 6-3 The mean (\pm 95% CI of the mean) Hb concentration increment (Hb concentration at time t minus pre-transfusion Hb concentration) per unit of RCC transfused for patients in the main study group (n=21).



There was no association between the storage age of the RBC units and the 24-hour recovery determined by single and dual label techniques ($r = 0.19$ and 0.41 respectively).

6.5 Discussion.

The results of this prospective study indicate that the 24-hour recovery of leucodepleted allogeneic RBCs in critically ill patients is approximately 91%. This was well within the currently accepted standard of a mean in-vivo recovery of 75%.

6.5.1 Critique of study methodology.

Measuring the recovery of stored RBCs is technically difficult. This is illustrated by a study that found high inter-laboratory differences in reported 24-hour recovery of concentrated RBCs stored for 35 days in citrate-phosphate-dextrose-adenine. The laboratory means of 24-hour recovery ranged from 45 to 85%.¹⁹⁰ This extent of variability was attributed to lack of uniformity in the methods employed. A survey of the literature highlights that the methods used to determine the 24-hour recovery of RBCs with either the single-label or dual-label techniques vary widely. In addition substantial variability exists with regard to the analysis of the data generated. A well-accepted dual-label technique was used. Established RBC labelling techniques were used.^{188;189} The data were analysed using 2 different methods, a single label and a dual label technique.

The single-label technique is certainly simpler than the dual-label technique but it can overestimate RBC recovery. The 24-hour recovery is a ratio of the count rate at 24 hours and at time-zero (100% survival value). The count rate at time zero cannot be measured and must be estimated. If this value is underestimated the RBC recovery will be overestimated. The early time averaged value method uses the mean of the early count rates (5 to 15 minute samples) as the 100% recovery value. This method ignores any loss that occurs in the first 5 minutes post-injection and therefore may underestimate the true time-zero value leading to an overestimation of 24-hour recovery. There are other techniques used to estimate the time zero value such as the maximum value method and the back extrapolation method. The maximum value method uses the maximum count rate of the early (5 to 15 minute) samples as the 100% recovery value. The main concern with this technique is that it relies on a single "outlier" value. The count rate at time-zero can also be estimated by drawing a best fit straight line through the data points obtained between 5 and 15 minutes post-injection using a logarithmic regression program and extrapolating back to time-zero. The problem with this approach is that it assumes that the rate of loss of label between 0 and 5 minutes is the same

as that between 5 and 15 minutes, when blood sampling is conducted. It has been speculated that early loss of RBCs maybe more rapid because storage-damaged RBCs may not survive more than one passage through the spleen. In addition, this method is particularly reliant upon complete blood mixing, which normally occurs within 3 minutes in healthy individuals but may take longer in certain disease states such as splenomegaly, cardiac failure and perhaps critical illness. In some of the patients the count rates were found to increase over the 5 to 15 minute period, which is consistent with delayed mixing of the radiolabelled RBCs within the patient's RBC pool. Rapid early loss and delayed mixing may both result in an underestimation of the true time-zero value.

Dual-label techniques are reportedly more accurate than single-label techniques because they are less affected by early loss of label and the effect of random blood loss e.g. bleeding. Using a second radionuclide to label autologous RBCs should permit a more accurate estimation of the true time-zero value because autologous RBCs should not undergo early loss. In addition, it allows the subsequent survival of allogeneic RBCs to be compared to the survival of autologous RBCs. However, the dual label technique is undoubtedly more complicated than the single label technique because it requires the use of two RBC labels. ^{51}Cr has many of the properties of an ideal label for RBC survival studies; its long 28-day half-life is convenient for RBC survival studies, in the doses employed it is chemically non-toxic and the effective dose of radiation is relatively small. Its main drawback is that it gradually elutes from RBCs as they circulate, but fortunately the eluted label is not reutilised. The choice of the second label is less obvious. The principal radiolabels used for the random labelling of RBCs are shown in Table 6-5. $^{99\text{m}}\text{Tc}$ is commonly employed as the second label for post-transfusional studies but it is far from ideal for the longer RBC survival studies. Firstly, the short half-life (6 hours) of $^{99\text{m}}\text{Tc}$ means that by 48 hours the count-rate from a sample is low or un-recordable. The 48-hour recovery could not be estimated by the dual-label technique in this study because the count-rates were too low. Administering a very

high activity overcomes this problem but this obviously entails greater radiation exposure for the patient. Secondly, ^{99m}Tc has a high and variable rate of elution. The main determinant of elution is thought to be the labelling process itself. The rate of ^{99m}Tc elution was measured in a separate group of critically ill patients (the elution group). The data obtained from the elution group was used to calculate a correction factor and then the correction factor was used to adjust the ^{99m}Tc count rates in the main study group. Twenty nine percent of ^{99m}Tc had eluted by 24 hours, which gave a correction factor of 1.41, which is similar to that found in other studies (1.55).¹⁹¹ The true value of ^{99m}Tc elution may have been slightly higher than this because no corrections were made for ^{51}Cr elution (see below); any loss of ^{51}Cr label was attributed to actual blood loss and the ^{99m}Tc count rate was corrected accordingly. However, using a correction factor derived from a mean rate of elution of ^{99m}Tc meant that in some patients the 24 hour ^{99m}Tc count rates were over-corrected and in others they were under corrected; this would account for the greater dispersion of the 24-hour recovery estimates seen with the dual-label technique.

Table 6-5. Radionuclide labels for the random labeling of RBCs.

Radiolabels	Principal radiation	Half-life ($t_{1/2}$)	Rate of elution	Effective dose (mSv)	Comments
Sodium chromate ^{51}Cr	γ	27.8 days	Approximately 1% per day although may be greater in first 24 hours.	0.5	^{51}Cr liberated by elution or RBC destruction is not re-utilised.
Pertechnate ^{99m}Tc	γ	6 hours	Very variable	0.02	Able to be counted accurately because of its high rate of decay but this results in a short half-life and needs to be counted quickly.
Indium chloride ^{111}In	γ	67.2 hours	Approx. 6-15% in the first 24 hours. The eluted label accumulates in plasma.	1.5	Complex labelling technique because it is not taken up selectively by RBCs (binds to WBCs, platelets and transferrin).
Di-isopropyl fluorophosphonate ^{32}P	β	14.3 days	5-10% in the first 24 hours.	High because of β emission.	Binds to cholinesterases in RBCs, other cells and in plasma.
^{14}C cyanate	Weak β emitter	5700 years	Cyanate binds irreversibly to Hb and elution is minimal		Its long half-life means that counting is very tedious and less accurate compared to ^{51}Cr . For these reasons it would be impractical for post-transfusion recovery studies.

Despite these limitations and potential sources of error, the 2 methods gave similar values for 24-hour recovery so it is unlikely that there was significant under- or over- estimation of the true red cell survival in these critically ill patients. However, the fact that the single and dual label techniques gave similar results raises into question the justification for performing the dual label technique. It was a much more complex study, it involved greater radiation exposure for the patients, it introduced further sources of error, which was reflected in the larger SD, and yet the mean RBC post-transfusional recovery was very similar.

The current standard for RBCs of a mean in-vivo recovery of 75% 24-hours after infusion refers to RBC units at the end of their storage life; in the UK the current RBC storage limit is 35 days. The mean storage age of the RBC units transfused in this study was 22 days (range 4-29 days). The 24-hour recovery of transfused RBCs is known to decrease with increasing duration of storage.¹⁸⁴ Although no such association was found in this study, it was not powered for this end-point. No attempt was made to influence the storage age of the transfused blood because it was not considered feasible for the blood bank to provide compatible “near” 35 days red cells units at short notice when there was a pressing clinical need. The storage ages of the RCCs used in this study are representative of the storage ages of RCCs transfused in a larger audit of blood use in Scottish ICUs.¹⁹²

The count-rates reported here have not been corrected for ⁵¹Cr elution. Ignoring ⁵¹Cr elution will result in an underestimation of 24-hour recovery because any loss of label will be attributed to loss of labelled RBCs. The degree of underestimation will be proportional to the rate of elution. ⁵¹Cr elutes at a rate of approximately 1% per day¹⁹³ although loss in the first 24 hours maybe slightly greater.¹⁹⁴ Studies have also shown that the rates of elution may vary significantly between different individuals¹⁹⁵ and with different labelling techniques.¹⁹³ It was not felt necessary to correct the count rates for ⁵¹Cr elution in this study because the labelling technique involved washing the labelled cells prior to injection to remove any loosely bound label and ⁵¹Cr elution is only a significant cause of label loss when other

losses are minimal (i.e. 24-hour recovery is high) or RBC survival studies are conducted over several weeks.

There are no currently accepted standards for RBC survival beyond 24 hours, therefore this study was limited to the first 48 hours following transfusion. A longer follow-up would have incurred a high dropout rate due to repeat transfusions and patient death. In addition, radiolabel elution would also have assumed a greater significance with a longer follow-up.

The Hb increment in this study was greater than that reported in a previous audit.¹⁸¹ This could be explained by differences in the Hb content of the RCCs used in this study and the previous audit, however, it could also be due to bias in the selection of patients for the present study. In this study patients were recruited and then transfused the following day, this was to allow time to prepare the radiolabels. This may have inadvertently led to the selection of more stable patients who could wait overnight for their transfusion.

6.5.2 Comparison with other studies.

This is the first study of allogeneic red cell recovery in critically ill patients. Studies of 35-day-old non-leucodepleted CPD/SAGM red cell concentrates in healthy volunteers have found 24-hour recovery estimates of 83 to 87.5%.¹⁸⁴ The data suggest that critical illness, and sepsis in particular, does not have a significant affect on allogeneic red cell viability, at least over the first 48 hours following the transfusion.

6.5.3 Implications of the findings.

These results do not justify the use of fresh red cells if the aim is to provide better RBC viability. These results do not infer anything about RBC function, however, determining the 24-hour recovery of transfused allogeneic RBCs in patients is a prerequisite for the assessment of the efficacy of blood transfusion. A much-quoted study found that 28-day-old rat blood failed to improve systemic oxygen consumption (VO_2) in supply dependent rats in contrast to fresh rat blood.¹¹³ A subsequent study found that rat RBCs deteriorate much more

rapidly during storage than human RBCs and that after 28 days of storage only 5% remain viable.¹¹⁴

The red cell product used in this study is representative of that used in many countries around the world. Universal leucodepletion of blood products is now standard practice in the UK, Canada, France, Germany, Ireland, New Zealand and Portugal, and it is being increasingly adopted in the US. Additive solutions, such as SAGM, are also widely used as they permit more of the plasma from the original donation to be utilised in the manufacture of other blood products. SAGM is the principal additive solution used in the UK and it is licensed for the storage of RBCs for up to 35 days. Other additive solutions are available, some of which, such as Adsol Preservation Solutions 1 and 3, are licensed for the storage of RBCs for up to 42 days. SAGM and Adsol differ only in the amounts of glucose, adenine and mannitol they contain. The other main red cell product variable is obviously the storage age; the range of storage age of the red cell units used in this study is typical of clinical practice.

In conclusion, these findings demonstrate that leucodepleted red cell concentrate suspended in saline-adenine-glucose-mannitol with a storage age ranging from 10 to 29 days has very good 24 and 48-hour recovery in critically ill patients.

7 The in-vivo regeneration of red cell 2,3 diphosphoglycerate following the transfusion of stored blood to critically ill patients.

7.1 Abstract.

The transfusion of stored 2,3 diphosphoglycerate (2,3 DPG) depleted blood is probably of little clinical significance to the majority of recipients because they are well enough to compensate for it. The clinical consequences of a transfusion of 2,3 DPG depleted blood to critically ill patients may be more significant because of their reduced physiological reserve. The regeneration of 2,3 DPG was determined following the transfusion of stored group O blood to group A critically ill patients.

There was a wide range of pre-transfusion 2,3 DPG concentrations in the critically ill patients; the mean (SD) pre-transfusion 2,3 DPG concentration was 12.6 (5.3) $\mu\text{mol/g Hb}$. Normal values for our laboratory were 14 (1.7) $\mu\text{mol/g Hb}$. The mean (SD) 2,3 DPG concentration of the RCCs was 2.6 (3.4) $\mu\text{mol/g Hb}$, or 16% of the recipient's pre-transfusion 2,3 DPG concentration. Four hours post-transfusion the whole blood 2,3 DPG concentration in the critically ill patients had decreased by a mean of 1.3 $\mu\text{mol/g Hb}$ per unit RCC transfused. The stored allogeneic RBCs regenerated 2,3 DPG relatively rapidly. The 2,3 DPG concentration of the recovered RBCs was 55% of the estimated 2,3 DPG concentration of the recipient's autologous RBCs at 4 hours, 76% at 24 hours and 93% at 72 hours post-transfusion.

The implications of these findings on tissue oxygenation are not known but any difference in the efficacy of fresh and stored RBCs should be most marked within the first few hours following transfusion.

7.2 Introduction.

The clinical significance of transfusing stored 2,3 DPG depleted blood is not known.¹⁹⁶ It is hypothesised that blood with increased oxygen affinity would release less oxygen at the periphery.

In an isolated heart model myocardial oxygen consumption and myocardial contractility were reduced when the heart was perfused with 2,3 DPG depleted blood compared to blood with a normal 2,3 DPG concentration.¹⁹⁷ In a study of patients undergoing coronary artery bypass operations, transfusion of blood with 2,3 DPG concentrations 150% of normal resulted in a higher post-bypass oxygen consumption and both the arterio-venous oxygen content difference and cardiac output increased.¹⁹⁸ They assumed that these responses were secondary to the improved contractile state of the myocardium as a result of improved oxygen delivery. In an animal model of anaemic shock resuscitation with 2,3 DPG depleted blood was associated with a significantly higher cardiac output and lower oxygen consumption.¹⁹⁹

In the majority of transfusion recipients the 2,3 DPG concentration of the blood is probably of little significance because they are able to compensate for it (see Section 2.7). The clinical consequences of a transfusion of 2,3 DPG depleted blood to critically ill patients may be more significant because of their reduced physiological reserve.

Several studies in healthy volunteers and other patient groups have shown that the 2,3 DPG concentration of transfused blood is restored to normal or near normal but the reports vary as to the rate and degree of regeneration (Table 7-1).

Table 7-1 Summary of the literature pertaining to the in-vivo rate of recovery of 2,3 DPG in stored blood.

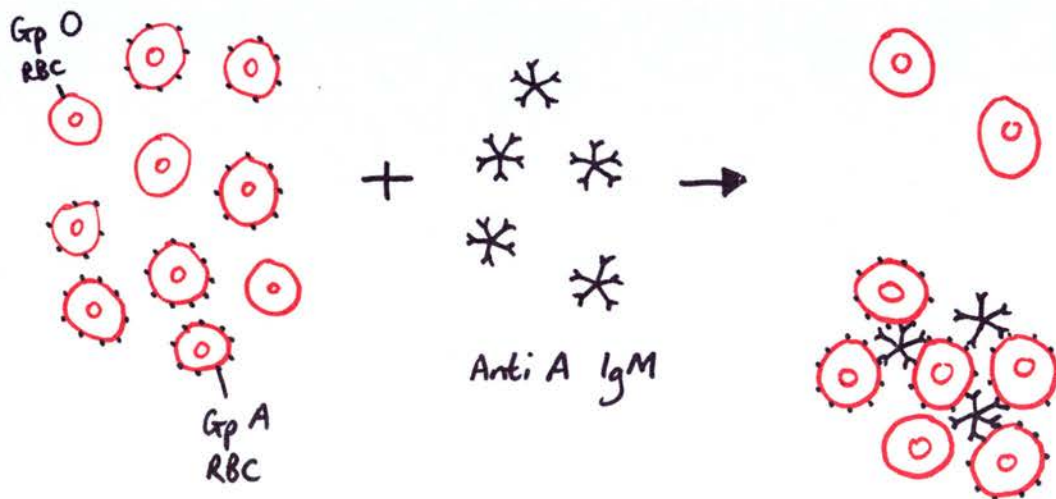
Study	Subjects	Transfused blood	Measurements	Recovery rate
200	Anaemic patients (n=2)	400 mL stored (35 days) RBCs	Recovered blood 2,3 DPG	50% of pre-storage 2,3 DPG within 1 hour, 100% by 2 hours.
121	Anaemic patients (n=3)	ACD stored (17 to 20 days) packed RBCs	Recovered blood 2,3 DPG	Approx. 50% of normal by 4 hours, almost normal by 24 hours.
201	Combat casualties (n=31)	≥12 units each of fresh (<1 day) and/or stored (14 days) WB	WB 2,3 DPG	50% of normal immediately post-transfusion, 75% by 12 hours and within normal range by 48 hours (normal = 11.9 (2.2) $\mu\text{mol/g Hb}$)
120	Healthy volunteers (n=5)	Stored (35 days) RBCs in additive solution	Recovered blood 2,3 DPG	Approx. 50% of peak 2,3 DPG at 7 hours. Peaked at 48-72 hours (89% of units pre-storage 2,3 DPG or 95% of recipient's pre-transfusion 2,3 DPG).
122	Anaemic non-surgical patients (n=3)	Stored (15 to 16 days) WB or packed RBCs	Recovered blood 2,3 DPG	25% of recipient's pre-transfusion 2,3 DPG by 3 hours, 50% by 24 hours but took 11 days to reach 100%.
202	Cardiac surgery patient (n=1)	4500 mL of stored blood. Storage time not specified	p50	Left shift took more than 4 days to correct.
119;203	Massively transfused patients (n=5)	9 to 35 units. Storage time and RBC product not specified	WB 2,3 DPG	50% recovery in 4 to 24 hours
204	Anaemic paediatric oncology patients (n=95)	Stored RBCs in additive solution	WB 2,3 DPG	Patients who received chemotherapy at the same time as or just after transfusion did not show a restoration of 2,3 DPG over several weeks

Key: Recovered blood Recovered by differential agglutination (predominantly allogeneic RBCs)

7.3 Aim:

To investigate the in-vivo regeneration of red cell 2,3 DPG following the transfusion of stored blood to critically ill patients. A technique, called differential agglutination, was used to recover the transfused allogeneic RBCs.^{205,206} This technique involves the transfusion of compatible group O blood to group A recipients. After transfusion, blood samples are taken and incubated with anti-A antibody to remove the recipient's autologous RBCs and recover the transfused group O RBCs (Figure 7-1). Flow cytometry was used to determine the proportion of group A RBCs in the whole blood samples taken post-transfusion, and to determine the degree of contamination of the recovered group O RBCs with the recipient's autologous group A RBCs.

Figure 7-1 Differential agglutination using IgM anti A antibody to agglutinate (and therefore remove) group A red blood cells from a mixed suspension of group O & A cells. Most group O red cells remain in the suspension although some are trapped in the agglutinate. Similarly, some group A red cells escape agglutination and remain in the suspension (contaminants).



7.4 Materials and methods.

The study was approved by the local research ethics committee.

7.4.1 Patient selection.

Ventilated and sedated critically ill patients who required a blood transfusion were eligible for recruitment. The decision to transfuse was made by the ICU consultant looking after the patient. The following inclusion/exclusion criteria were used:

Inclusion criteria.

Haemoglobin concentration <90 g/L.

Blood group A.

Exclusion criteria.

Patients who showed evidence of blood loss or were haemodynamically unstable.

Blood group O, B or AB.

Informed assent not obtained.

7.4.2 Recruitment.

The patients' next of kin were approached for informed assent.

7.4.3 The blood transfusion.

The patients were crossmatched with group O RCC units. Each RCC unit was given over 1 hour using a volumetric pump and blood-warming device.

7.4.4 Blood sampling.

- 1) Prior to the transfusion 10 mL of the patient's whole blood was collected into ACD anticoagulant and a 10 mL sample was taken from each RCC unit.

- 2) At 4, 24 and 72 hours after the completion of the transfusion 25 mL of whole blood was collected into ACD anticoagulant.

7.4.5 Sample processing.

The 2,3 DPG concentration of each sample was determined using a commercially available kit (SIGMA) (see Section 4.4.3). This value represents the whole blood (WB) 2,3 DPG concentration.

The remainder of the 25 mL ACD samples taken at 4, 24 and 72 hours were then subjected to differential agglutination.

Differential agglutination.

The differential agglutination technique used here is essentially the same technique described by Winifred Ashby nearly 100 years ago.²⁰⁵ The technique had previously been validated, by the principal investigator, to ensure that it did not interfere with the 2,3 DPG measurements (see Section 10.4 Appendix 10.4). The sample was centrifuged and the supernatant discarded. Forty mL of murine monoclonal IgM anti-A antibody (Diagnostic Scotland, Plasma Fractionation Centre, Ellen's Glen Road, Edinburgh, UK) was added to the packed RBCs. The mixture was then left for 1 hour at 4°C. The mixture was shaken gently, centrifuged at 200 g for 5 minutes and then allowed to settle for 5 minutes; this process was repeated twice in all. The mixture was then tapped gently and put on ice for 10 minutes. The mixture was tapped again and put on ice for a further 5 minutes after which the supernatant was decanted. The separation technique took approximately 2 hours.

The amount of Hb recovered in the supernatant was estimated by multiplying the volume of the recovered sample by its Hb concentration. The volume of recovered blood was calculated by multiplying the net weight of the sample by 1.056 (the specific gravity of RBCs). The Hb concentration of the supernatant was measured using a Sysmex FBC analyser.

The 2,3 DPG concentration of the recovered RBCs was measured as described earlier.

An aliquot of the recovered RBCs was fixed for flow cytometry (see Section 7.5.2 below) to determine the degree of contamination with residual group A RBCs.

The 2,3 DPG concentration of the recipient's autologous RBCs was estimated using Equation 7-1:

Equation 7-1

$$\begin{aligned} \text{2,3 DPG of WB sample} = & \text{2,3 DPG of recipient's RBCs} \times \text{proportion of A-positive RBCs} \\ & + \\ & \text{2,3 DPG of recovered RBCs} \times (\text{proportion of A-negative RBCs} - \\ & \text{contaminants}) \end{aligned}$$

Key:

WB whole blood

7.4.6 Data analysis.

Demographic data, diagnostic data and sequential organ failure assessment (SOFA) scores were recorded for all of the patients. ICU outcome (survivor versus non-survivor) was also recorded. The data are expressed as mean (SD), unless stated otherwise. A General Linear Model Repeated Measures ANOVA (SPSS version 11.5.0) was used to compare the 2,3 DPG concentrations between survivors and non-survivors. The within-patient coefficient of variation of the recipient's autologous RBC 2,3 DPG concentration was also determined.

7.5 Flow cytometry.

7.5.1 Blood sampling protocol.

An aliquot of the recipient's pre-transfusion blood sample (A-antigen positive control) and an aliquot of blood from each of the group O RCC units (A-antigen negative controls) were obtained.

Aliquots of whole blood and recovered RBCs were fixed for flow cytometry as outlined below.

7.5.2 RBC fixation.

Fixed erythrocytes were prepared by adding 100 μL of packed RBCs to 1 mL phosphate buffered saline (PBS) containing 50 $\mu\text{g}/\text{mL}$ sodium dodecyl sulphate (SDS) and 1 mg/mL bovine serum albumin (BSA). After one minute 9.7 mL PBS, 0.3 mL formalin (37% formaldehyde in PBS) and 10 $\mu\text{g}/\text{mL}$ SDS was added. After 90 minutes, 0.8 mL of formalin was added and left at room temperature overnight. The cells were washed in PBS and the procedure was repeated excluding the addition of BSA. The fixed erythrocytes were finally washed in PBS and re-suspended in 1 mL PBS containing 5 mg/mL BSA and 0.1% sodium azide.

7.5.3 Red cell labelling.

The fixed erythrocytes were diluted with PBS containing 5 mg/mL BSA and 0.1% sodium azide to a concentration of 2×10^7 cells/mL. Fifty μL (approximately 10^6 RBCs) of fixed erythrocytes was added to 50 μL anti-A IgM (murine IgM immunoglobulin, Diagnostic Scotland, Plasma Fractionation Centre, Ellen's Glen Road, Edinburgh, UK) and incubated for 30 minutes at room temperature. The cells were washed twice and then incubated with 50 μL anti-murine IgM FITC labelled antibody (Star86F, Serotec Oxford, UK) for 30 minutes. The cells were then washed twice in PBS/BSA/azide and re-suspended in 1 mL PBS. The cells were then ready for analysis by flow cytometry.

7.5.4 Flow-cytometric analysis.

Labelled fixed RBCs were analysed on a Coulter FACS analyser. The RBC population was gated on their forward scatter and side scatter characteristics. To confirm that the cells being analysed were RBCs samples were labelled with an IgG antibody directed against the RBC

antigen Glycophorin A (BRIC 256, IBGRL, Bristol, UK). Glycophorin A is a marker of erythroid cells and is one of the most abundant antigens found on red blood cells. 50 000 cells were counted per sample.

A-antigen positive (the pre-transfusion sample of the group A recipient's RBCs) and A-antigen negative (a sample of the transfused group O RBCs) controls were included with each analysis.

This indirect immunofluorescence flow cytometry technique had been validated by the principal investigator (see Section 10.5 Appendix 10.5).

7.6 Results

Eight patients were recruited. The patient details are shown in Table 7-2.

7.6.1 Pre-transfusion 2,3 DPG concentrations.

The 2,3 DPG concentration of stored blood and the storage times are shown in Table 7-3.

The mean pre-transfusion 2,3 DPG concentration was 12.6 (SD 5.3) $\mu\text{mol/g Hb}$ (Table 7-4).

7.6.2 Post-transfusion 2,3 DPG concentrations.

The 2,3 DPG concentration of WB and the recovered group O RBCs following transfusion is shown in Table 7-4. Following transfusion there was an immediate decrease in the WB 2,3 DPG concentration in all of the patients; four hours post-transfusion the WB 2,3 DPG concentration had decreased, compared to the pre-transfusion level, by a mean of 1.32 $\mu\text{mol/g Hb}$ per unit of RCC transfused. The trend in the WB 2,3 DPG concentration over the next 3 days was very variable (Figure 7-2). The trend in the estimated 2,3 DPG concentration of the recipient's autologous RBCs was also very variable; the mean within-patient coefficient of variation of the recipient's estimated autologous RBC 2,3 DPG concentrations (pre-transfusion to 72 hours) was 16.3%.

Table 7-2 Patient details.

Pt ID	Age	Sex	Diagnosis	Previous		ICU LOS (days)	SOFA score	ICU outcome
				transfusion (days previously)	APACHE II score			
1	40	M	Septic shock	N	21	6	10	Died
2	37	M	Respiratory failure	N	19	2	3	Survived
3	49	M	Acute pancreatitis	N	13	5	4	Survived
4	76	M	ARDS	N	27	29	8	Survived
5	45	F	Other GI surgery (laparotomy sepsis)	N	33	9	15	Died
6	50	F	Septic shock	Y (5)	27	21	12	Survived
7	79	M	Pneumonia-bacterial	N	25	5	8	Died
8	75	M	Community acquired pneumonia	Y (5)	36	29	10	Died

Key: APACHE II Acute physiology and chronic health evaluation II score 24 hours after admission to the ICU.

ICU LOS Intensive care unit length of stay.

SOFA score Sequential organ failure assessment score at enrolment to the study (does not include a score for the central nervous system because this was difficult to assess due to the presence of sedation).

There was a statistically significant difference between the WB 2,3 DPG concentrations of ICU survivors and non-survivors ($p = 0.022$).

The 2,3 DPG concentration of the recovered group O RBCs increased relatively rapidly after transfusion. The mean (SD) 2,3 DPG concentration of the recovered RBCs at 4, 24 and 72 hours post-transfusion was 5.6 (2.8) $\mu\text{mol/g Hb}$, 7.8 (4.7) $\mu\text{mol/g Hb}$ and 9.4 (6.5) $\mu\text{mol/g Hb}$ respectively. When the 2,3 DPG concentration of the recovered group O RBCs was expressed as a percentage of the estimated 2,3 DPG concentration of the recipient's autologous RBCs at that same timepoint, the corresponding mean values were a 55% recovery at 4 hours, 76% at 24 hours and 93% at 72 hours (Figure 7-3).

7.6.3 Differential agglutination performance.

The amount of Hb recovered by differential agglutination was very variable. A mean of 30.7 (SD 15.5) % of the amount of group O Hb estimated to be present in the original 25 mL sample was recovered. The contamination rates were low and most were less than 2% (Table 7-4), although one sample (the 24-hour sample, patient 4) had a very high contamination rate (37.7%).

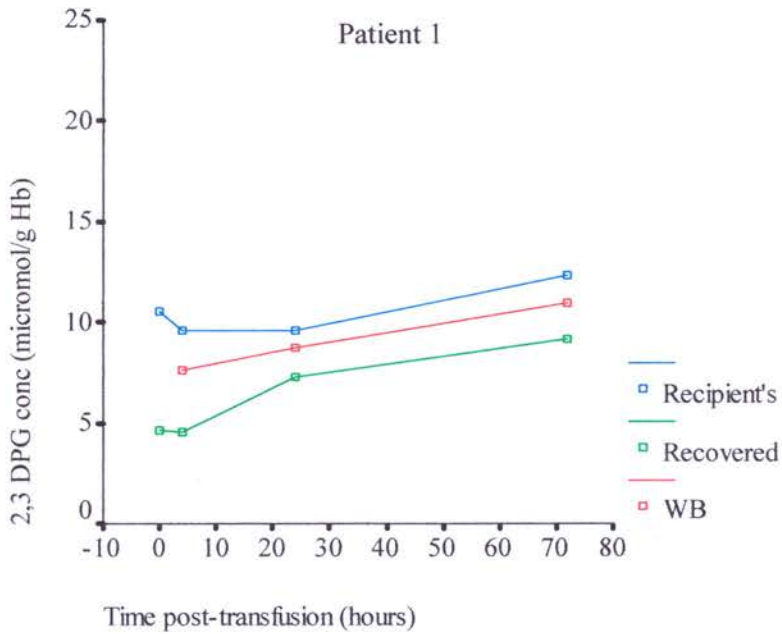
Table 7-3 The storage time, blood group and 2,3 DPG concentration of the RCC received by each patient.

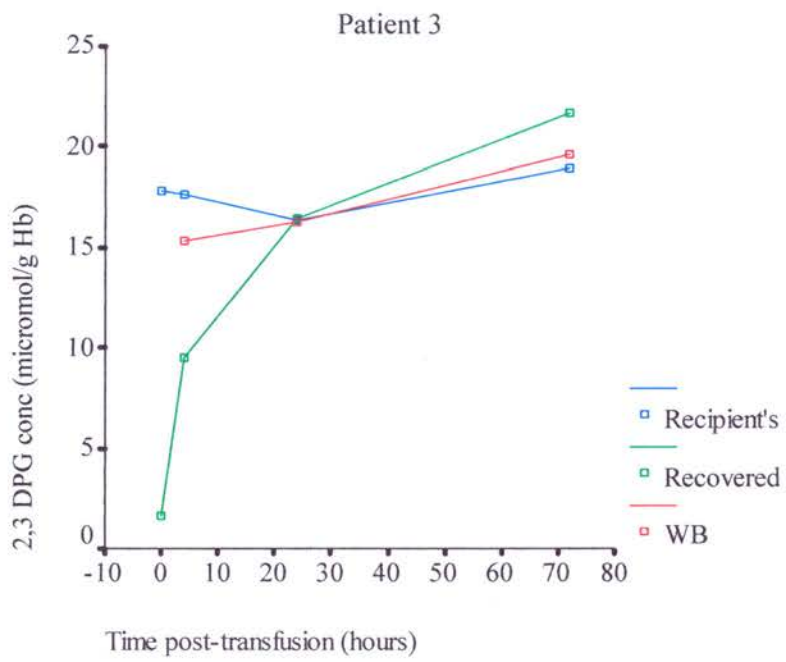
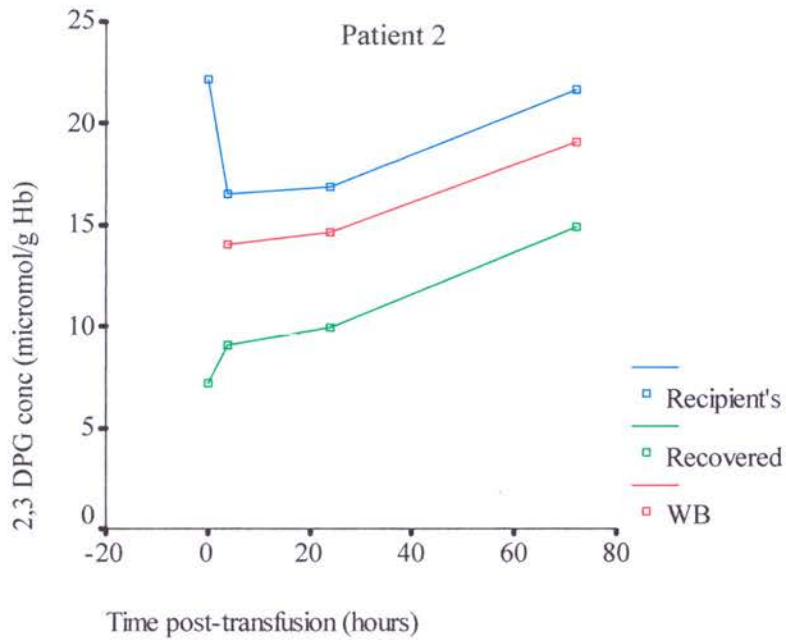
Patient	No of units	ABO Rh	Storage time (days)	2,3 DPG concentration ($\mu\text{mol/ g Hb}$)
1	2	A Pos	10	1.2
			10	8.0
2	3	A Pos	3	11.5
			7	2.3
			5	7.8
3	2	A Pos	13	1.6
			13	1.7
4	2	A Pos	11	4.0
			10	2.7
5	2	A Neg	29	0.7
			29	0.5
6	2	A Pos	24	0
			24	0
7	2	A Pos	18	0.2
			17	0.4
8	2	A Neg	30	0.5
			29	0.6

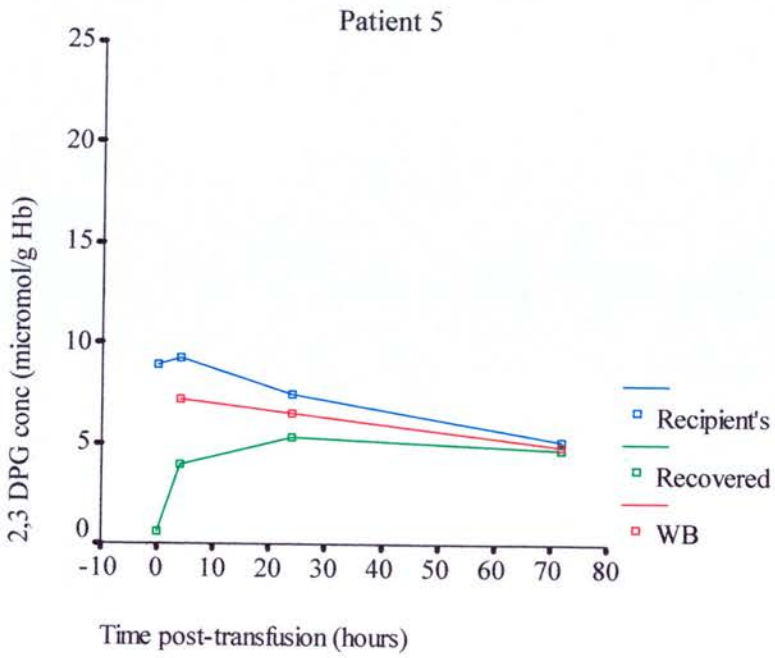
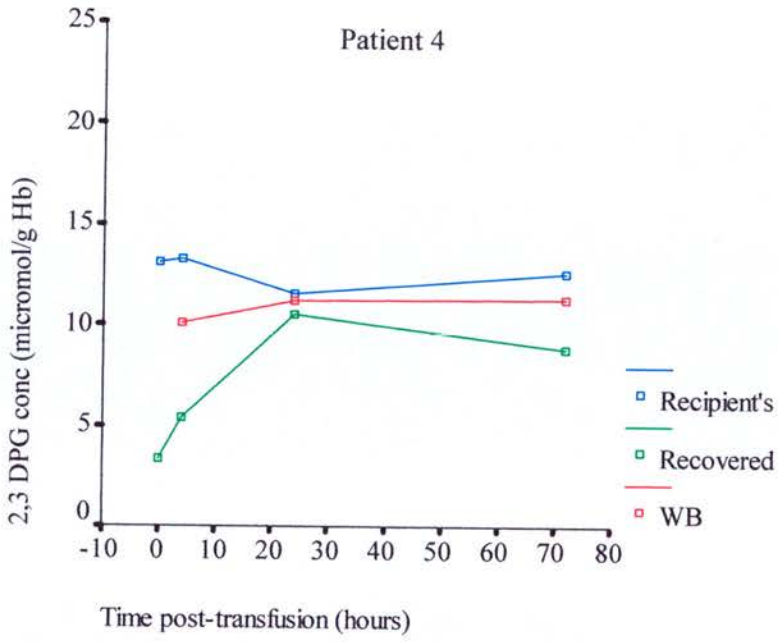
Table 7-4 The 2,3 DPG concentration of whole blood (WB) and recovered RBCs at 4, 24 and 72 hours. The 2,3 DPG concentration of a whole blood sample taken from the recipient pre-transfusion (pre-Tx) is also reported. The percentage of A-antigen positive RBCs present in the recovered RBCs is reported as contamination.

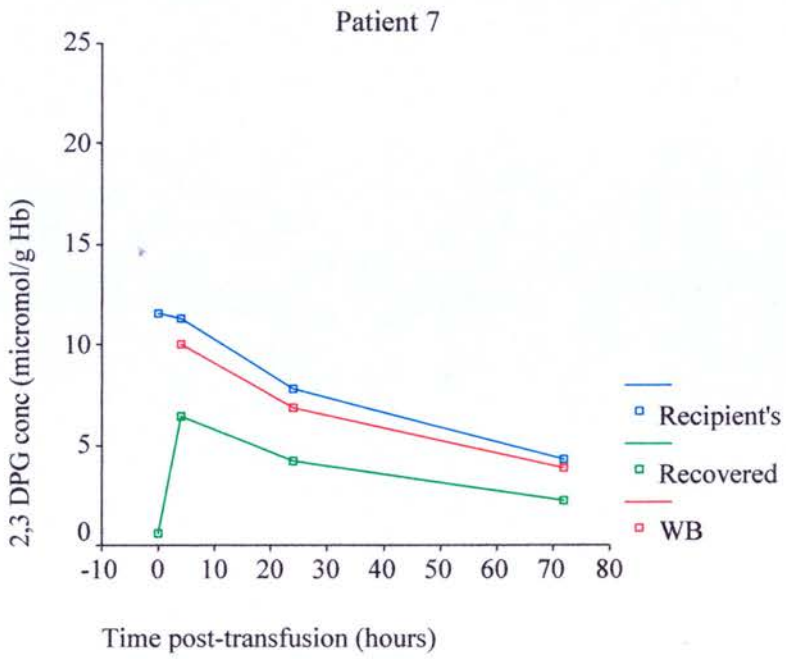
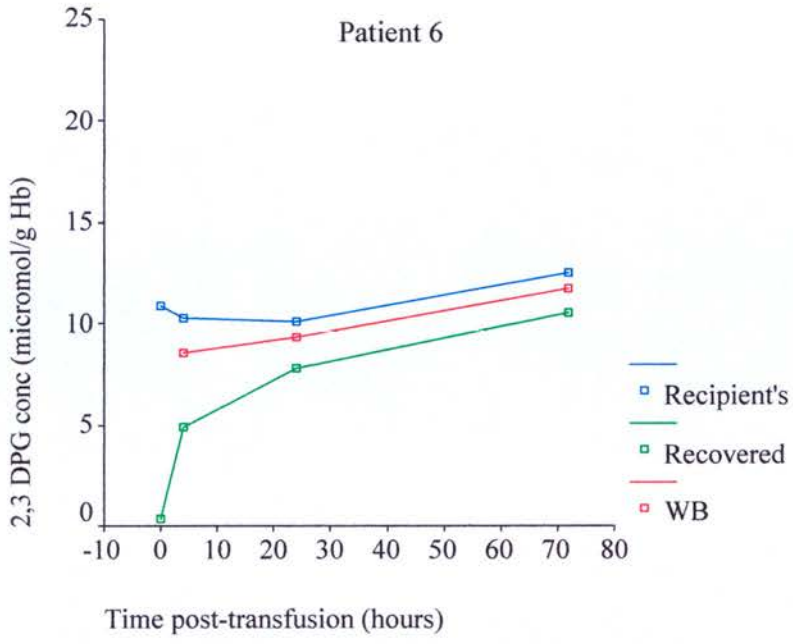
Patient ID	Blood sample	2,3 DPG concentration ($\mu\text{mol/g Hb}$) pre-transfusion (pre-Tx) and at 4, 24 and 72 hours after blood transfusion			
		Pre-Tx	4 hours	24 hours	72 hours
1	WB	10.5	7.6	8.7	11.0
	Recovered RBCs		4.5	7.3	9.2
	Contamination		0.95%	0.51%	0.58%
2	WB	22.2	14.0	14.6	19.1
	Recovered RBCs		9.1	9.9	14.9
	Contamination		1.1%	0.45%	0.88%
3	WB	17.8	15.3	16.3	19.6
	Recovered RBCs		9.5	16.4	21.7
	Contamination		2.9%	1.1%	1.1%
4	WB	13.1	10.1	11.2	11.3
	Recovered RBCs		5.4	10.6	8.8
	Contamination		4.3%	37.7%	3.4%
5	WB	8.9	7.2	6.5	4.9
	Recovered RBCs		3.9	5.3	4.7
	Contamination		0.45%	0.3%	1.7%
6	WB	10.9	8.6	9.3	11.7
	Recovered RBCs		4.9	7.8	10.5
	Contamination		1.2%	4.4%	3.1%
7	WB	11.6	10.0	6.8	3.9
	Recovered RBCs		6.4	4.2	2.2
	Contamination		0.3%	0.2%	0.6%
8	WB	5.4	3.8	2.6	2.0
	Recovered RBCs		0.95	0.96	3.0
	Contamination		0.2%	0.3%	0.3%

Figure 7-2. The 2,3 DPG concentration of whole blood and recovered group O RBCs measured at 4, 24 and 72 hours for patients 1 – 8 (overleaf). The 0 hour values correspond to the pre-transfusion measurements; the data point for the 2,3 DPG concentration of the recovered group O RBCs at 0 hours represents the 2,3 DPG of the RCC. The estimated 2,3 DPG concentration of the recipient's autologous RBCs is represented by the blue line.









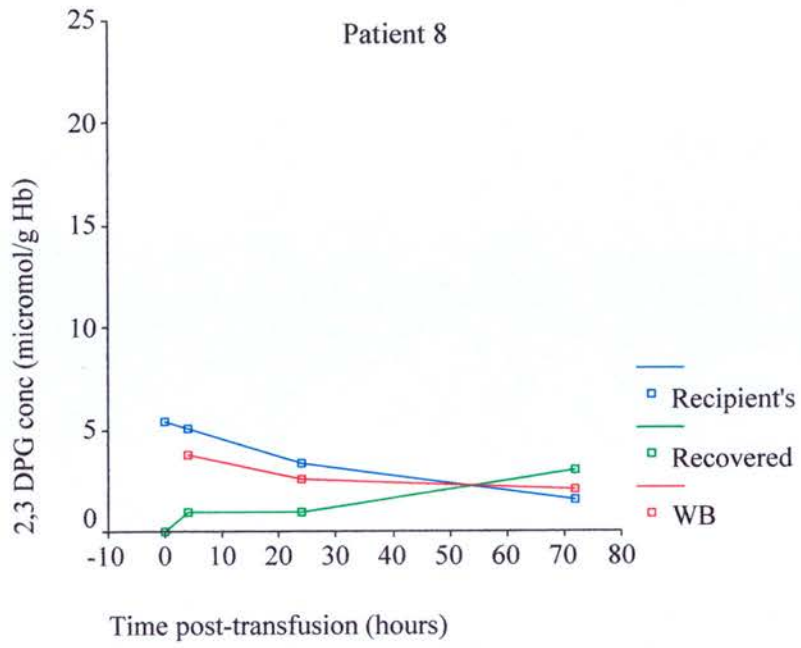
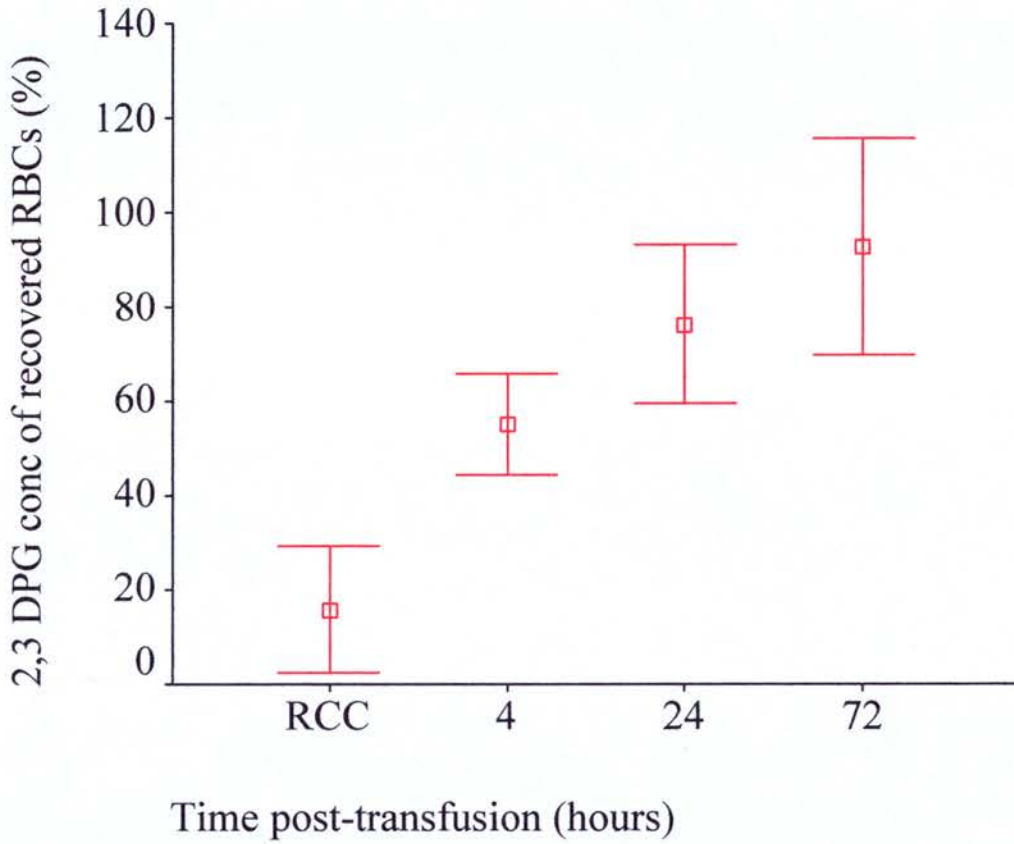


Figure 7-3 The 2,3 DPG concentration of the recovered group O RBCs at 4, 24 and 72 hours expressed as a percentage of the estimated 2,3 DPG concentration of the recipient's autologous RBCs at that timepoint (the chart displays the mean \pm 95% confidence interval of the mean).



Mean (%)	15.7	55	76.3	92.6
95% CI of the mean (%)	4.3 to 27.1	45.9 to 64.1	62.3 to 90.6	73.4 to 111.8

7.7 Discussion.

This prospective study found that stored blood regenerates 2,3 DPG relatively rapidly following transfusion to critically ill patients and approaches the 2,3 DPG concentration of the recipient's autologous RBCs by about 72 hours.

Although only 8 patients were recruited to this study it is still larger than most of the previous studies looking at 2,3 DPG regeneration. There are a number of factors that contributed to the low recruitment rate. For obvious reasons recruitment was restricted to patients of blood group A; blood group A is prevalent in approximately 42% of the UK population. The study required group A patients to be crossmatched with group O blood, due to the other clinical commitments of the hospital blood bank this could only be performed between the hours of 9 am and 5 pm. In addition only one patient could be recruited at any one time because of the onerous nature of the laboratory techniques. Finally, and most importantly, production of the 2,3 DPG assay kits was permanently discontinued.

The 2,3 DPG concentrations of the stored RCCs were typical with respect to their storage times (see Figure 4-10).

The critically ill patients recruited to this study were representative of the UK ICU population; they were elderly, there was a predominance of males and they had common ICU diagnoses. Only 2 of the patients had received a blood transfusion previously during their admission; in both cases the transfusions had been administered 5 days prior to enrolment. Although the mean pre-transfusion autologous 2,3 DPG concentration was 12.6 $\mu\text{mol/g Hb}$, which is within the normal range for our laboratory (mean 14.0 (SD 1.7) $\mu\text{mol/g Hb}$), there was marked variability (SD 5.3 $\mu\text{mol/g Hb}$). Previous studies that have measured the RBC 2,3 DPG concentration in critically ill patients have produced conflicting results. One study found that critically ill patients with acute respiratory distress syndrome (ARDS) had higher RBC 2,3 DPG concentrations (mean 19.9 (SD 3.9) $\mu\text{mol/g Hb}$) than control

subjects (mean 12.5 (SD 2.1) $\mu\text{mol/ g Hb}$).⁴⁸ These ARDS patients were severely hypoxaemic with a mean PaO_2 5.7 kPa and they had a tendency to alkalaemia (pH 7.44). Conversely, a more recent study in Australia found that critical illness was associated with a reduced RBC 2,3 DPG concentration (mean 4.2 (SD 1.3) mmol/L) compared to control subjects (mean 4.9 (SD 0.5) mmol/L).⁴⁹ None of the patients in this second study were hypoxaemic and they had a slight tendency to acidaemia (pH 7.37). The different PaO_2 and pH findings probably explain the different 2,3 DPG concentration results of these two studies since prolonged severe hypoxaemia and alkalaemia can both increase 2,3 DPG concentrations.²⁰⁷ A much older study, reported in 1970, also looked at the RBC 2,3 DPG concentration during critical illness. The study consisted of 11 patients with characteristic signs and symptoms of septic shock. The report is lacking in details, but one of the more interesting findings was “that the 2,3 DPG level appeared to mirror the patients’ general clinical status; the poorer the clinical criteria the lower the 2,3 DPG and further left the p50; with significant improvement in clinical status the 2,3 DPG approached and often overshot normality.”²⁰³ In fact, no patient with a 2,3 DPG concentration less than 2 $\mu\text{mol/mL}$ survived. The present study also found an association between low 2,3 DPG concentrations and non-survival.

The 2,3 DPG concentration of the WB and the estimated 2,3 DPG concentration of the recipient’s autologous RBCs varied markedly over the subsequent 3 days. This is in keeping with an earlier observational study performed in our unit (unpublished data courtesy of Dr Ezz el din Saleh Mohamed Ibrahim). The primary aim of that study was to determine what happens to the RBC 2,3 DPG concentration during critical illness. The secondary aim of the study was to investigate the factors that may influence RBC 2,3 DPG concentration during critical illness. Daily 2,3 DPG measurements were performed. The data presented in Table 7-5 are from a cohort of 48 critically ill patients who remained in the ICU for 72 hours after enrolment and who did not receive a blood transfusion. There was a wide range of 2,3 DPG

concentrations (Table 7-5). Over the subsequent 72 hours the 2,3 DPG concentrations varied markedly, the within-patient standard deviation was 3.7 $\mu\text{mol/L}$. This is greater than the within-patient standard deviation of the patient's whole blood 2,3 DPG concentration, of 1.96 $\mu\text{mol/L}$, in this present study. This implies that other factors associated with critical illness have a much greater effect on the 2,3 DPG concentration than the transfusion of 2-3 units of stored RCC. The study found a highly significant correlation between 2,3 DPG and arterial pH ($r^2=0.47$, $p<0.0001$) and weaker correlations with creatinine ($r^2=-0.35$, $p<0.0001$), potassium ($r^2=-0.2$, $p=0.032$), phosphate ($r^2=-0.2$, $p=0.046$), chloride ($r^2=-0.19$, $p=0.044$), and glucose ($r^2=0.19$, $p=0.044$). There were no significant correlations between 2,3 DPG and Hb, blood lactate, oxygenation indices, other electrolyte concentrations, age or temperature. Regression analysis was then used to construct a model to describe/predict the 2,3 DPG concentration based on all the measured parameters. Only pH and the chloride concentration appeared in the final regression equation. The regression coefficients for pH and chloride were 32.2 (95% CI 19.1 to 46.2) and -0.196 (95% CI -0.39 to -0.01) respectively. These data suggest that 2,3 DPG had the strongest relationship with pH and a weak association with chloride. The model only explained about 29% of the observed variation in RBC 2,3 DPG.

There were insufficient data points from the present study to investigate accurately which parameters influence the 2,3 DPG regeneration of stored blood.

Table 7-5 Sequential RBC 2,3 DPG concentrations in critically ill patients (unpublished date courtesy of Dr Ezz el din Saleh Mohamed Ibrahim).

Time (hours)	2,3 DPG concentration ($\mu\text{mol/g Hb}$) of autologous RBCs	
	Present study (n=8) Mean (SD)	Previous study (n=48) Mean (SD)
0	12.6 (5.3)	14.6 (7.0)
24	10.4 (4.5)	14.7 (6.4)
72	11.1 (7.1)	17.4 (7.1)

The rate of 2,3 DPG regeneration was relatively rapid. The 2,3 DPG concentration of the stored RBCs increased to approximately 55% of the 2,3 DPG concentration of the recipient's autologous RBCs by 4 hours, 76% at 24 hours and 93% by 72 hours. The time course for the in vivo replenishment of 2,3 DPG in this study is not inconsistent with the results of the studies of less sick subjects (Table 7-1). Most of the published studies are very small (n=1 to 3) and are quite old using blood collection and storage methods that are now obsolete. Some of the studies used a combination of packed RBCs and whole blood, and blood units with very different storage times within the same patient. Comparisons between studies are also difficult because the studies used different methodologies; where small volumes of blood were transfused differential agglutination was performed, whereas when large volumes of blood were transfused (effectively an exchange transfusion) WB 2,3 DPG was measured.

This study has found that it takes a relatively short, but finite, time for stored RBCs to regenerate their 2,3 DPG. The time course for the 2,3 DPG regeneration in critically ill patients appears comparable to that found in other less sick patient groups and healthy volunteers. Whether or not the transfusion of 2,3 DPG depleted blood has a detrimental effect on critically ill patients remains to be elucidated. Further studies are required to

investigate the effects of stored blood transfusion on tissue oxygenation, focusing in particular on the first few hours post-transfusion.

8 Red blood cell survival determined by flow cytometry.

8.1 Abstract.

Post-transfusional RBC survival is one of the recommended criteria for the assessment of the quality of blood storage. However, RBC survival studies are difficult to perform and therefore, they are conducted infrequently. A measure of RBC survival post-transfusion is a necessary prerequisite to the assessment of the efficacy of blood transfusion. The aim of this study was to develop a flow cytometric technique that could be used to perform allogeneic RBC survival studies in the clinical setting. Blood group A critically ill patients and haematology patients with chronic anaemia were recruited. They were transfused crossmatched group O blood. Serial blood samples were obtained post-transfusion. Indirect immunofluorescence was performed against the A-antigen to determine the proportion of allogeneic RBCs in the samples.

In the critically ill patients (n=8) the proportion of allogeneic RBCs remained relatively constant over the 72-hour study period. This suggests that stored allogeneic and autologous RBCs have similar viability in critically ill patients.

In the haematology patients (n=4), the RBC disappearance slope was linear over the 12-week study period. Extrapolation beyond 12 weeks revealed that "fresh" (storage time ≤ 5 days) allogeneic RBCs had a median (range) lifespan of 104 (86 to 124) days.

This study demonstrated that flow cytometry can be used to provide clinically useful RBC survival data.

8.2 Introduction.

RBC survival following transfusion is one of the recommended criteria for the assessment of the quality of blood storage. Current standards, for RBCs, require a mean in vivo recovery of $\geq 75\%$ 24 hours after infusion.¹⁸⁵ There are no recommendations concerning RBC survival beyond 24 hours. This is mainly because the estimation of RBC lifespan is technically difficult. Studies of RBC survival usually involve measuring the rate of disappearance of labelled RBCs from the circulation. The problems with this approach are well documented; the RBC label can elute,²⁰⁸ the eluted label can “re-label” other RBCs and the label itself may have adverse effects. The “ideal” method for the estimation of RBC survival would be one that accurately measures the fate of the transfused RBCs rather than a surrogate RBC label.

Flow cytometry can accurately quantify antigenically distinct cell populations. Flow cytometry could be used to track the fate of transfused allogeneic RBCs, avoiding the problems of RBC labels.

8.2.1 Aim

To develop a flow cytometric technique that could be used to perform RBC survival studies in the clinical setting.

8.3 Materials and methods.

Deliberate antigenic differences between donor and recipient RBCs were used to perform RBC survival studies in two groups of patient:

- a. Critically ill patients
- b. Haematology outpatients

8.3.1 Critically ill patients.

The cohort of patients studied in Chapter 7 also had blood samples taken to determine RBC survival. EDTA samples were taken from each RCC (A-antigen negative control RBCs) and from each patient before (A-antigen positive control RBCs) and at 0, 4, 24, 48 and 72 hours after the transfusion.

8.3.2 Haematology outpatients.

Blood group A patients attending a haematology day-case unit for RBC transfusion, and who were likely to require further RBC transfusions, were eligible for recruitment. Patients of blood groups O, B & AB, and patients less than 16 years were excluded from the study. Informed consent was sought from eligible patients by the principal researcher. After informed consent had been obtained arrangements were made for the study to be initiated at the patient's next attendance for RBC transfusion. The timing of that transfusion and the number of RCC units to be transfused were decided by the physician looking after the patient. Patients were crossmatched for group O RCCs that had been donated no more than 5 days previously. Prior to the transfusion a 2.7 mL EDTA sample of whole blood was taken from the patient (A-antigen positive control RBCs) and a 2 mL aliquot was obtained from each RCC unit (A-antigen negative control RBCs). Each RCC was then given over 2.5 hours via an infusion pump. After completion of the transfusion an EDTA sample was taken from the patient at 15 minutes, 24, 48 and 72 hours and then twice weekly thereafter for up to 7 weeks. 2 further blood samples were taken at approximately 80 and 84 days.

8.3.3 Sample processing.

A full blood count was performed on each blood sample (Sysmex analyser).

A small aliquot of each blood sample was taken for chemical fixation. The sampling technique that was used to obtain the aliquots was modified whilst the study was still ongoing in an attempt to minimise measurement error:

- a. The blood samples from the first 4 critically ill patients were centrifuged at 200 g for 5 minutes and a 100 μL aliquot of packed RBCs was aspirated from the RBC pellet. One aliquot only was taken from each blood sample.
- b. The blood samples from the subsequent critically ill patients were mixed using a vortex mixer and a 200 μL aliquot of RBC suspension was aspirated from each sample. Again, only one aliquot only was taken from each blood sample.
- c. The blood samples obtained from the haematology outpatients were mixed using a vortex mixer and three 200 μL aliquots of RBCs in suspension were aspirated from each sample.

Each sample was then fixed, labelled and analysed by flow cytometry as described earlier (see Sections 7.5.2, 7.5.3 and 7.5.4). 50 000 cells were counted per sample.

8.3.4 Controls.

A-antigen positive (a pre-transfusion sample of the group A recipient's autologous RBCs) and A-antigen negative (a sample of the transfused allogeneic group O RBCs) controls were included with each analysis.

8.3.5 Data analysis.

Critically ill patients.

A General Linear Model Repeated Measures ANOVA (SPSS version 11.5.0) was used to analyse the change in the proportion of group O RBCs over the 72-hour study period and the difference between the two fixation methods (pellet versus suspension).

Haematology outpatients.

The proportion of the Hb concentration in the patient's sample that was contributed by the group O RBCs was calculated by multiplying the Hb concentration by the mean proportion of group O RBCs. The Hb concentration of the group O RBCs was plotted against time. The

Pearson correlation coefficient (r) was determined as a measure of how closely the data points assumed a straight line. A least squares linear regression analysis was performed (SPSS version 11.5.0). Linear extrapolation to an Hb concentration of zero g/L was performed to obtain an estimate of RBC lifespan.

8.4 Results.

8.4.1 Critically ill patients.

The proportion of group O RBCs detected by flow cytometry over the 72-hour post-transfusion period for patients 1 to 4 and patients 5 to 8 is shown in Figure 8-1 and Figure 8-2 respectively. A preliminary review of the data was conducted after obtaining the results from the fourth critically ill patient. There appeared to be random variation in the proportion of group O RBCs in patients 2 and 4 (Figure 8-1). The RBC fixation technique was reviewed (see Section 10.6 Appendix 10.6) and subsequently modified; for patients 5 to 8 the blood samples were no longer centrifuged prior to aspirating the RBC aliquot for fixation. In this study there was no statistically significant difference between the 2 fixation methods ($p=0.61$) but the mean within-patient coefficient of variation was greater when the sample was taken from the RBC pellet (8.8%) rather than from the RBC suspension (4.9%).

The Hb increment expressed as a percentage of the post-transfusion Hb and the percentage of group O RBCs are shown in Table 8-1 and Figure 8-3 (A).

Figure 8-1 The proportion of group O RBCs present in the recipient's circulation 15 minutes, 4, 24, 48 and 72 hours post-transfusion. For patients 1 – 4 the RBC sample for fixation consisted of a 100 μ L aliquot of packed RBCs aspirated from the RBC pellet.

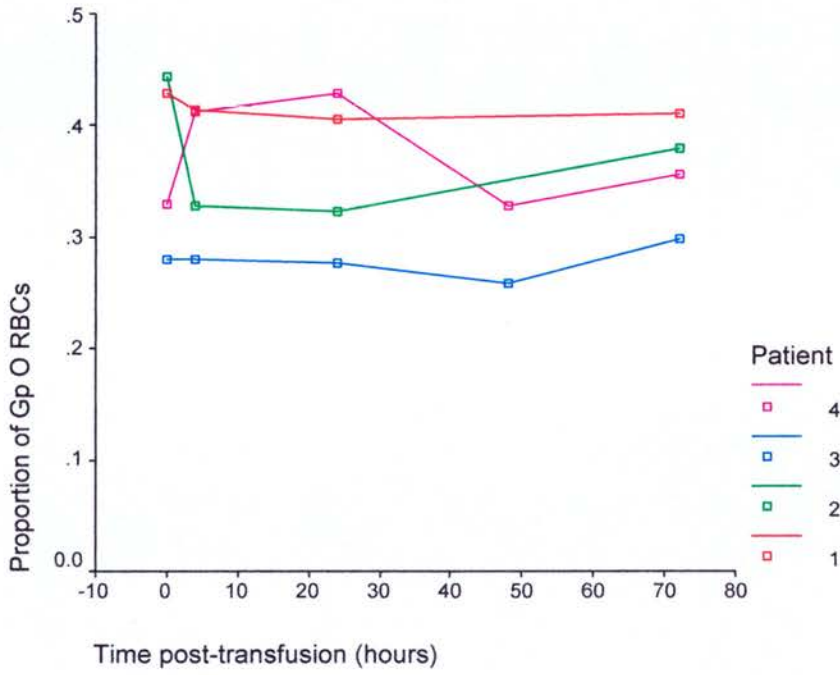


Figure 8-2 The proportion of group O RBCs present in the recipient's circulation 15 minutes, 4, 24, 48 and 72 hours post-transfusion. For patients 5 – 8, the RBC sample for fixation consisted of 200 μ L of RBC suspension.

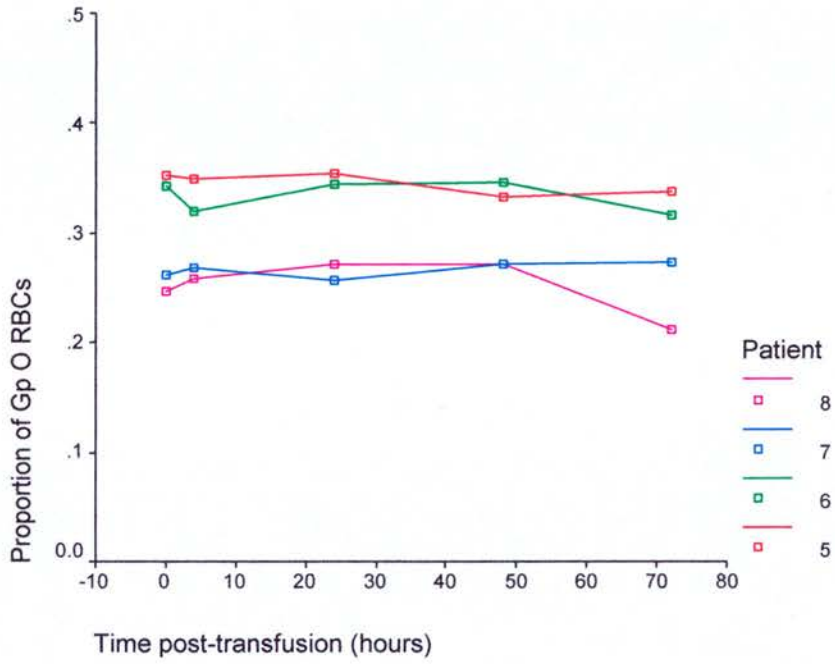


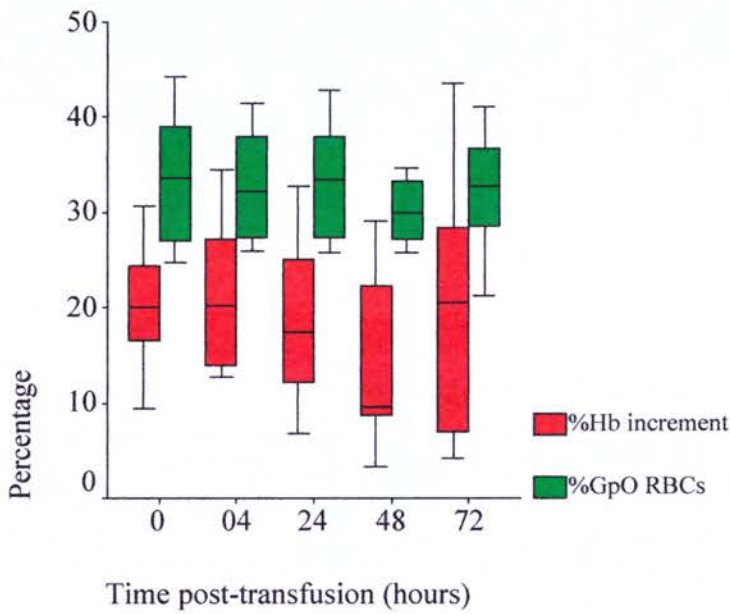
Table 8-1. The Hb increment ([Hb – pre-transfusion Hb]/Hb*100) and the percentage of group O RBCs.

Patient	Time post-transfusion (hours)						Mean (SD)	Median (IQR)
	0	4	24	48	72			
1	Hb increment (%)	30.7	34.6	32.7		43.5	35.4 (5.6)	33.7 (32.2-36.8)
	GpO RBCs (%)	42.8	41.4	40.5		41.1	41.5 (1.0)	41.3 (41-41.8)
2	Hb increment (%)	21.4	22.4	25.0		34.7	25.9 (6.1)	23.7 (22.1-27.4)
	GpO RBCs (%)	44.3	32.7	32.3		37.9	36.8 (5.6)	35.3 (32.6-39.5)
3	Hb increment (%)	9.4	14.3	13.5	10.3	5.0	10.5 (3.7)	10.3 (9.4-13.5)
	GpO RBCs (%)	27.9	27.9	27.6	25.8	29.8	27.8 (1.4)	27.9 (27.6-27.9)
4	Hb increment (%)	18.8	12.8	6.8	8.9	8.9	11.2 (4.8)	8.9 (8.9-12.8)
	GpO RBCs (%)	33.0	41.2	42.8	32.8	35.6	37.1 (4.7)	35.6 (33-41.2)
5	Hb increment (%)	21.2	28.4	21.2	29.1	19.6	23.9 (4.5)	21.2 (21.2-28.4)
	GpO RBCs (%)	35.3	34.9	35.4	33.2	33.8	34.5 (1.0)	34.9 (33.8-35.3)
6	Hb increment (%)	27.5	25.8	25.0	22.4	21.4	24.4 (2.5)	25.0 (22.4-25.8)
	GpO RBCs (%)	26.2	26.9	25.7	27.2	27.4	26.7 (0.7)	26.9 (26.2-27.2)

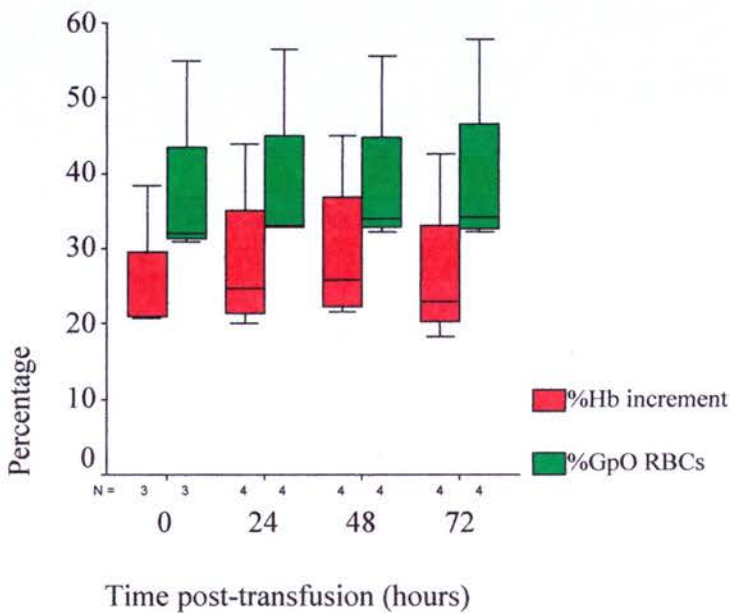
Patient		Time post-transfusion (hours)					Mean (SD)	Median (IQR)
		0	4	24	48	72		
7	Hb increment (%)	15.9	13.5	10.9	3.2	4.3	9.6 (5.6)	10.9 (4.3-13.5)
	GpO RBCs (%)	24.7	25.9	27.1	27.1	21.2	25.2 (2.4)	25.9 (24.7-27.1)
8	Hb increment (%)	17.1	18.2	13.7	8.7	22.2	16.0 (5.1)	17.1 (13.7-18.2)
	GpO RBCs (%)	34.2	31.9	34.5	34.6	31.6	33.4 (1.5)	34.2 (31.9-34.5)

Figure 8-3. Boxplot of the Hb increment expressed as a percentage of the post-transfusion Hb ($[\text{post-transfusion Hb} - \text{pre-transfusion Hb}] / \text{post-transfusion Hb} * 100$) and the percentage of group O RBCs in: (A) the 8 critically ill patients and (B) the 4 haematology outpatients.

(A) Critically ill patients.



(B) Haematology outpatients.



8.4.2 Haematology outpatients.

Patient details and the transfusion details are shown in Table 8-2.

Table 8-2 Patient details and storage time of red cell concentrates (RCC).

	Age	Sex	Diagnosis	RCCs	
				Number transfused	Storage time (days)
1	72	F	Myelodysplastic Syndrome	2	5
2	30	F	Thalassaemia	2	3
3	70	F	Myelodysplastic Syndrome	2	3
4	43	F	Diamond Blackfan Syndrome	3	3

A mean of 99.52 (SD 0.37)% of gated events were Glycophorin A positive. Good separation of the A-antigen positive and negative RBCs was obtained (Figure 8-4).

The Hb concentration profiles and the proportion of group O RBCs post-transfusion are shown in Figure 8-5 & Figure 8-6 respectively. A boxplot of the Hb increment following transfusion, expressed as a percentage of the post-transfusion Hb, and the percentage of group O RBCs is shown in Figure 8-3 (B).

Survival plots, including Pearson's correlation coefficient (r) and lifespan estimates of the transfused group O RBCs are shown in Figure 8-7. The median RBC lifespan was 104 (range 86 to 124) days.

Figure 8-4 Fluorescence histogram of a whole blood sample taken from a group A patient who had received a therapeutic transfusion of group O blood. Indirect immunofluorescence directed against the A antigen has been performed.

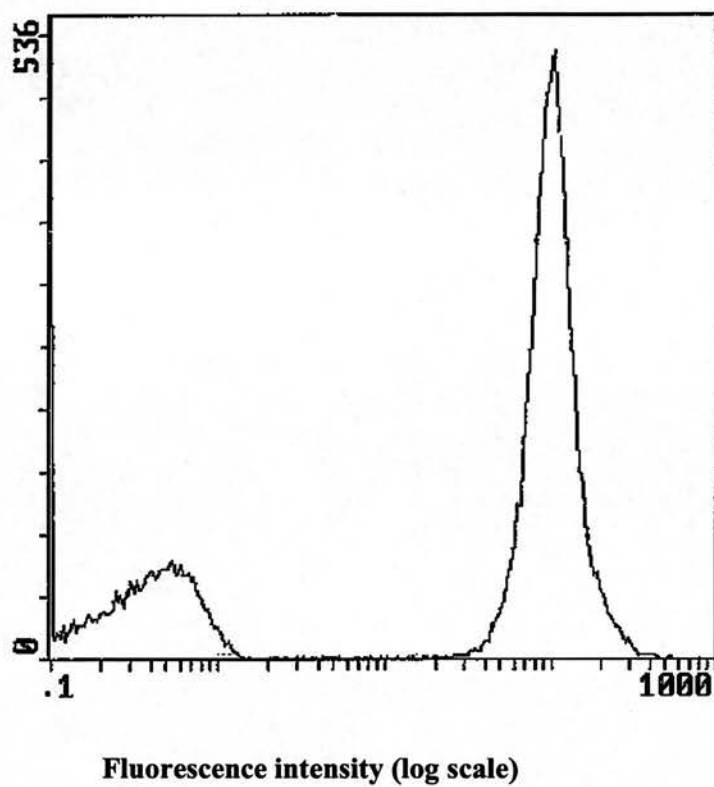


Figure 8-5 The Hb concentration profiles of the patients over the 7 weeks following the transfusion of group O blood (day 0).

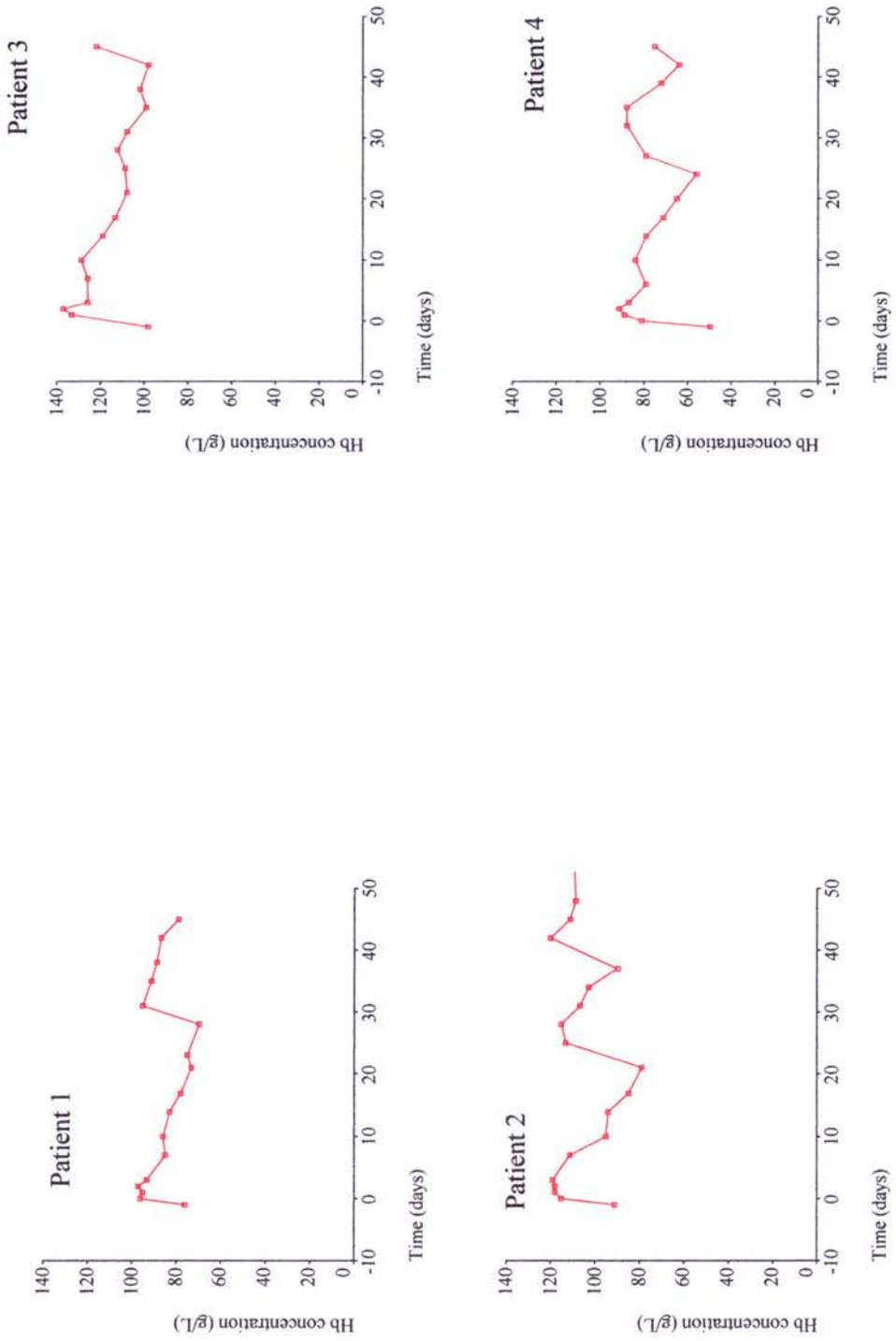


Figure 8-6 Plots of the proportion of group O RBCs in whole blood samples taken from the patients post-transfusion (group O transfusion - day 0, subsequent group A transfusions - indicated by arrows).

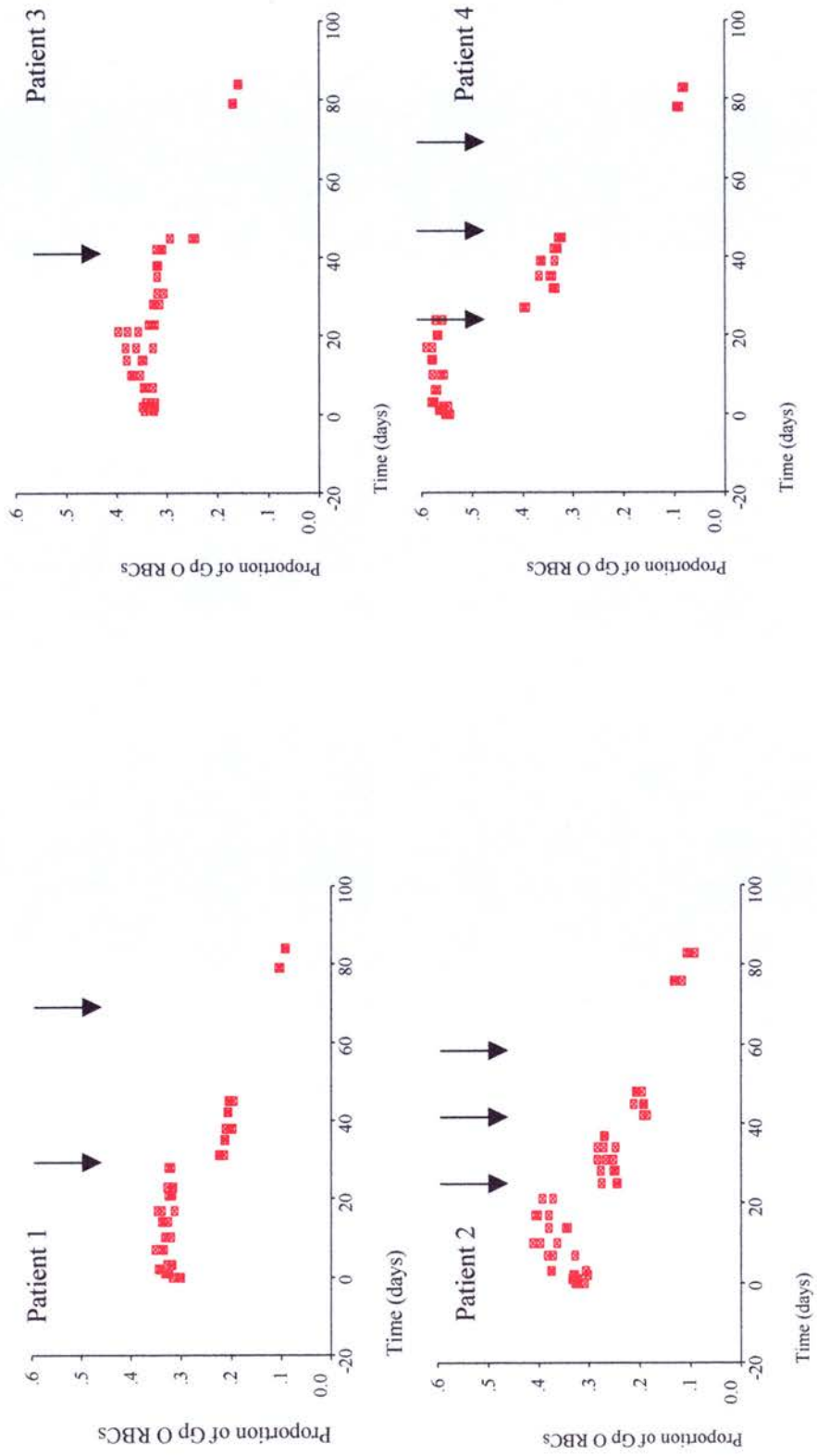
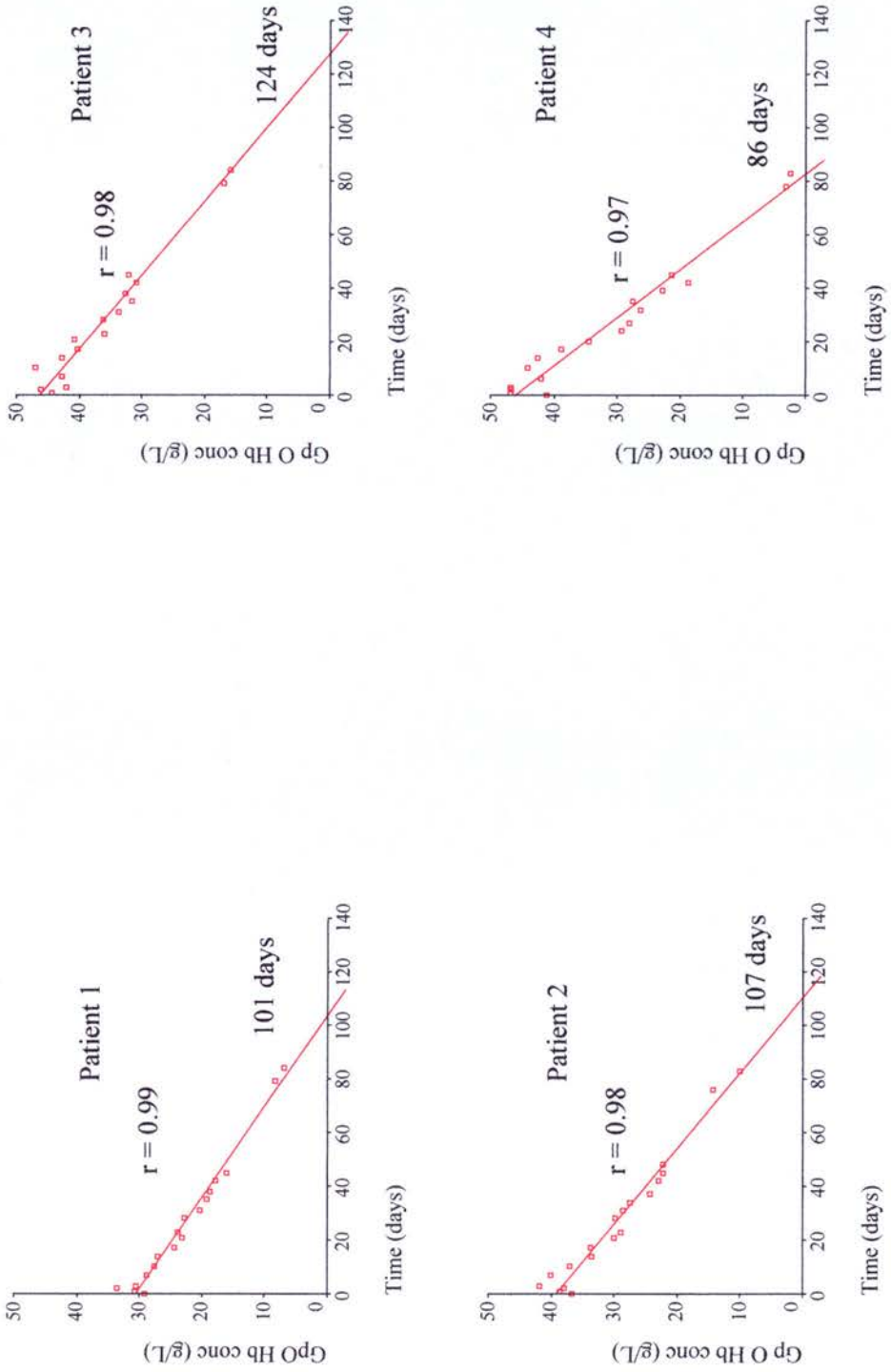


Figure 8-7 Allogeneic group O RBC disappearance slopes. Plots, for patients 1, 2, 3 & 4, of the proportion of the Hb concentration that is due to the group O RBCs (Hb concentration multiplied by the proportion of RBCs that are group O) against time. The intercept of the line with the x-axis represents the RBC lifespan



8.5 Discussion.

This study has demonstrated that flow cytometry can be used to perform allogeneic RBC survival studies in the clinical setting.

8.5.1 Critically ill patients.

The study in critically ill patients found that there was no significant change in the proportion of group O RBCs over the 72-hour study period. This suggests that the allogeneic and autologous RBCs had a similar viability. This does not support the hypothesis that stored allogeneic RBCs may undergo selective (non-random) destruction in critically ill patients.

A preliminary review of the results from the first 4 patients found that, in some patients, there was random variation in the proportion of circulating group O RBCs over the 72-hour study period. It was thought that this variability was most likely due to sampling error. This was tested (see Chapter 10.6 Appendix 10.5). The coefficient of variation was found to be greater when the aliquot of RBCs for fixation consisted of packed cells (from a pellet) rather than a RBC suspension. This is presumably due to uneven distribution of the fresh and stored RBCs within the pellet. The sampling technique was modified for all of the subsequent patients.

8.5.2 Haematology outpatients.

This study demonstrated that flow cytometry can be used to perform allogeneic RBC survival studies over several weeks. These patients were followed-up for approximately 12 weeks. The very high correlation coefficients suggest that the RBC disappearance slope, over the 12-week period, is linear. RBC lifespan was estimated by extrapolating the disappearance slope to the intercept with the x-axis. The median (range) RBC lifespan was found to be 104 (86 to 124) days. This is very similar to the quoted RBC lifespan of 110-120 days. This would appear to suggest that "fresh" RBCs (storage time ≤ 5 days) have a "near-normal" lifespan following transfusion.

Using antigenic differences between donor and recipient to track transfused RBCs is not a new idea. Differential agglutination was first described in 1911, in animals, and the technique was used in humans in 1919.^{205;209} The human study involved transfusing group O blood to group A recipients, taking blood samples and incubating them with anti-A serum; the A cells were agglutinated and the group O cells could then be counted. Early studies using this technique demonstrated that the erythrocyte had a long lifespan.^{205;210-213} Unfortunately there were also many reports of technical failures due to the lack of good quality agglutinating antibodies and difficulties quantifying the non-agglutinated group O RBCs. Differential agglutination was soon superseded by radio-isotopic methods.

The main advantage of radio-isotopic tracers is that they can be quantified with a high degree of accuracy, which is important when only small volumes of blood are administered.²¹⁴ In addition, radioisotopes can be used to label autologous blood. However radioisotopes obviously necessitate exposure to ionising radiation. Radio-iron required the use of unacceptably large amounts of radioactivity. Newer labels such as ⁵¹Cr involve much less radiation exposure. Other problems with the use of RBC labels are that the labelling process must be performed under sterile conditions and the label can wash-off or elute. Elution is particularly significant when estimating RBC lifespan where the studies are conducted over several weeks. Studies have shown that 50% of Cr elutes in just over 30 days and that the rate of elution is variable and unpredictable.²¹⁵⁻²¹⁷ To further complicate matters some labels, once eluted can be reutilised, or re-label other RBCs.

The advent of flow cytometry has provided the opportunity to revisit Ashby's technique. Using antigenic differences between donor and recipient RBCs is only applicable to allogeneic transfusions but these account for the majority of therapeutic transfusions. In theory any RBC antigen could be used for this technique. This present study used Ashby's original technique of transfusing group O blood to group A recipients because this technique has several advantages.²⁰⁵ The group A antigen is abundant ($1-2 \times 10^6$ per RBC) on the

RBC's surface and high affinity anti-A antibodies are readily available. These two factors determine the degree of separation of antigen positive and negative cells that can be obtained on the fluorescence histograms. Using anti-A IgM can cause agglutination and it was necessary to chemically fix the RBCs to prevent this. Obviously the technique is only applicable to individuals who possess the group A antigen but this represents 42% of the UK population. Finally, it is a safe technique; group O RBCs are safe for transfusion to persons of any ABO group.

All of the patients in this study received substantial volumes of allogeneic red cells. The aim of this study was to develop a technique that could be used to assess the efficacy of therapeutic transfusion and the use of large volumes of blood should increase the accuracy of the technique. Flow cytometric techniques have been developed that can detect much smaller amounts of allogeneic RBCs if required; these techniques have been used to quantify fetomaternal haemorrhage.²¹⁸⁻²²⁰

"Fresh" RBCs (storage age ≤ 5 days) were used in the haematology outpatient study to standardise the storage age of the RBCs and to minimise the effects of the red cell storage lesion on RBC survival.

The technique used in the haematology outpatients of plotting the group O Hb concentration against time will be imprecise if there are large fluctuations in the plasma volume. Fluctuations in plasma volume are well recognised as a cause of variability of the Hb concentration; such changes have been demonstrated in surgical patients during the perioperative period.^{4,5} The haematology outpatients were relatively well throughout the study period and significant fluctuations in plasma volume were unlikely to have occurred. Diurnal variation in the Hb concentration can occur; to minimise this effect all of the blood samples were taken at the same time of day. The very high r-values suggest that this was not a major source of error.

It is interesting to note that following the transfusion of group O blood there was a consistent discrepancy between the early Hb increment and the proportion of group O RBCs in a whole blood sample; the proportion of group O RBCs was always greater than the Hb increment and in some cases it was more than double that expected. This can only be assessed in the early samples (<72 hours) because later samples will have a lower Hb increment due to ongoing loss of autologous as well as allogeneic RBCs. This is an unexpected finding. It could be explained if the stored transfused RBCs contained less Hb than the recipients autologous RBCs but this is extremely unlikely. For this to be true the RBCs would have to contain less than half the Hb of the recipient's RBCs. The FBC indices, MCH and MCHC, of stored RBCs do not support this. Alternatively the flow cytometric technique may be under reading the group A RBCs and therefore over-reading the group O RBCs. However tissue controls (patient's group A RBCs and the transfused group O RBCs) were used in every case and the flow cytometric technique performed well in the validation. The anti A antibodies were of high affinity, they were used in sufficient concentration to produce intense fluorescence and good separation between A positive and A negative RBCs was obtained. Therefore the only remaining explanation would appear to be that the transfusion of stored red cell concentrate causes plasma volume to increase to a greater degree than the red cell mass; this too seems unlikely.

This technique can be used in the clinical setting, avoids exposure to ionising radiation, avoids the need for pre-transfusion (sterile) cell labelling and is not influenced by label elution. The lack of elution means that the technique is ideally suited for prolonged follow-up, which in turn will improve the accuracy of the estimate of red cell lifespan.

9 General discussion and conclusions.

Allogeneic RBC transfusion is an important supportive therapy for critically ill patients. Surprisingly, the efficacy and clinical effectiveness of RBC transfusion in this setting is still unclear. The recent European Commission's document "Blood safety in the European Community: an initiative for optimum use"²²¹ made the following statement concerning red blood cells:

"Patients require a product that has maximum oxygen delivery capability and minimised risk. As optimum storage time and conditions defined by clinical utility have never been determined they need to be urgently addressed"

Blood is an increasingly scarce resource, it is expensive to produce and transfusion carries significant risks. For some patients, there are alternatives to blood transfusion, such as not giving blood (restrictive transfusion strategy) or treatment with erythropoietin. There is a clear need for transfusion policies based on evidence of the efficacy and clinical effectiveness of blood transfusion.

9.1 The efficacy of allogeneic blood transfusion.

Most of the available evidence relates to earlier types of product such as whole blood (donor blood diluted with an anticoagulant solution with no attempt to remove plasma white cells or platelets before storage) that are now obsolete. The current red cell pack provided for transfusion has been processed to remove almost all the white cells and most of the plasma, resuspending the RBCs in a saline-adenine-glucose-mannitol solution. It cannot be assumed to have the same properties.

9.2 Red cell recovery after transfusion

A measure of RBC survival post-transfusion is a necessary prerequisite to the assessment of the efficacy of blood transfusion; lack of efficacy could be due to poor RBC viability. RBC

survival studies are difficult to perform and therefore infrequently conducted. There have been no RBC survival or recovery studies in critically ill patients. The present work has shown that, following transfusion to critically ill patients, stored RBCs have good short-term survival. Currently accepted standards, for RBC products, require a mean in vivo recovery of 75% or better 24 hours after administration. There are no recommendations for RBC survival beyond 24 hours, in part because it is widely assumed that if RBCs survive for 24 hours post-transfusion they will then have normal survival. Clearly when blood is administered it is hoped that its effects will persist well beyond 24 hours. More data are needed regarding longer-term RBC post-transfusional survival. The safe non-radioisotopic methods developed for this work, using RBC antigens as red cell labels, should encourage the performance of RBC survival studies in the clinical setting.

9.3 Tissue oxygenation.

Having proven that allogeneic RBCs survive in vivo the next step is to look for evidence of improved tissue oxygenation. The goal of RBC transfusion is to increase the haemoglobin concentration and consequently improve tissue oxygenation. Unfortunately it is difficult to reliably assess tissue oxygenation in the euvolaemic patient. This makes it difficult to measure the efficacy of RBC transfusion. A combination of whole body and organ specific parameters of tissue oxygenation are the minimum requirement. Patient morbidity and mortality should also be used as study endpoints, but this will obviously necessitate much larger studies. Animal models are useful because they permit the use of techniques, such as the estimation of tissue oxygenation by ruthenium fluorescence, that are not possible in the clinical setting. However extrapolating the results of animal experiments to the clinical setting is not always straightforward. Novel monitors that could be used in human studies to assess the effect of blood transfusion on tissue oxygenation include microdialysis catheters, and the commercially available Licox and Neurotrend devices. Microdialysis is being used to assess the composition of extracellular fluid within a variety of tissues. Microdialysis is

achieved via a fine coaxial catheter that can be sited within a tissue. The catheter has a dialysis membrane on its outer surface and low flow rates of dialysis fluid are passed through the catheter using a pump mechanism. Aliquots of fluid can be removed over a variable time period to allow measurement of substances within the extracellular fluid. On-line analysis is available but the fluid can also be taken to a remote machine for analysis. Elevation of the lactate/pyruvate ratio is being used as a marker of tissue ischaemia. The Licox device is a small polarographic Clark electrode that is being used primarily to assess brain PO_2 but it can be sited almost anywhere. The Neurotrend is a multi-parameter sensor that measures temperature, PO_2 , PCO_2 and pH using a fiberoptic probe: microvascular PO_2 is measured by a phenomenon known as "fluorescent quenching." Ideally multiple modalities should be monitored. By using continuous monitoring of more than one parameter, and preferably a combination of monitors of global and regional oxygenation, some of the limitations of each of the methods may be overcome. Multimodality monitoring may also help identify measurement errors from the different monitors because such errors will occur at different times with the different monitors. Unfortunately multimodality monitoring incurs greater costs in equipment, manpower and time and further increases the complexity of the studies.

9.4 The clinical significance of the red cell storage lesion.

There are specific concerns regarding the efficacy of stored, as opposed to freshly collected RBCs since they undergo marked changes during storage. The present studies on leucodepleted RBCs resuspended in SAGM confirmed that there was a rapid loss of 2,3 DPG during storage; only a slight decrease in RBC deformability was detected. Following transfusion to critically ill patients the stored allogeneic RBCs regenerated 2,3 DPG relatively rapidly. Four hours after transfusion the 2,3 DPG concentration of the allogeneic RBCs had risen from 16% of the estimated 2,3 DPG concentration of the recipient's autologous RBCs to 55%, and by 72 hours it was 93%.

The implications of these changes on tissue oxygenation are not known. The 2,3 DPG concentration of transfused RBCs recovers rapidly, this suggests that any difference in efficacy between fresh and stored blood should be most marked within the first few hours following transfusion. It is of great practical importance to determine if (or when) fresh RBCs could be superior to stored RBCs. More direct comparison studies between stored and fresh leucodepleted blood are needed. One such study conducted in our ICU merits detailed discussion. In this study critically ill patients were randomized prospectively to receive leucodepleted red cells that were either fresh (<5 days) or had prolonged storage time (>20 days). Ten patients received fresh red cells and 12 patients received stored red cells. Changes in gastric to arterial PCO_2 gap (P_g-PaCO_2 gap), gastric intramucosal pH, arterial pH, arterial base excess, and arterial lactate concentrations were measured during baseline (2.5 hours), during transfusion (3 hours), and for 5 hours after transfusion. There was no evidence of clinically relevant worsening in P_g-PaCO_2 gap, pHi , or any global measure of tissue hypoxia after transfusion of leucodepleted red cells stored for >20 days. Mean age of red cells stored <5 days was 2 days (range 2-3); red cells stored >20 days had a mean age of 28 days (range 22-32). The patient's haemoglobin concentration increased by 15.0 g/L and 16.6 g/L, respectively, in the fresh and stored groups ($p=0.62$). There were no significant differences between the groups either using treatment-by-time analysis or comparing the pre- and post-transfusion periods either for P_g-PaCO_2 gap (mean difference 0.03 kPa; 95% confidence limits 1.66 to 1.72) or gastric intramucosal pH (mean difference 0.015 pH units; 95% confidence limits 0.054 to 0.084). The mean change within each group from the pre- to post-transfusion period for P_g-PaCO_2 gap and gastric intramucosal pH, respectively, was 0.56 kPa (95% confidence limits 0.68 to 1.79) and 0.018 pH units (95% confidence limits 0.069 to 0.032) for "fresh" red cells and 0.52 kPa (95% confidence limits 0.6 to 1.64) and 0.033 pH units (95% confidence limits 0.080 to 0.129) for "stored" red cells. There was no statistically or clinically significant improvement in any other oxygenation index during the

measurement period for either group compared to baseline values. These findings do not support the use of fresh red cells in critically ill patients. Further such studies are needed, preferably using morbidity and mortality as outcome measures.

9.5 Blood collection, processing and storage.

The influence of RBC 2,3 DPG on the oxygen haemoglobin equilibration curve can be demonstrated *in vitro*. The effect of the RBC 2,3 DPG concentration on tissue oxygenation *in vivo* is less clear. RBCs rapidly lose 2,3 DPG with current UK blood collection, processing and storage methods. Alternative additive solutions, that maintain 2,3 DPG during storage, are available but they have not been widely adopted primarily because the clinical significance of RBC 2,3 DPG has not been demonstrated.

Loss of RBC deformability has been demonstrated during refrigerated storage. This study found a slight decrease in RBC deformability, relative to controls, after 35 days of refrigerated storage. Previous studies have documented larger changes. The clinical significance of RBC deformability is unclear. A loss of RBC deformability has been documented in many disease states, although whether this loss of deformability plays any role in the pathophysiology of the disease is not known. Further work is needed to determine which of the numerous assays of RBC deformability is the most appropriate for the measurement of RBC deformability during storage. Adequate assays are a prerequisite of studies that could elucidate the clinical significance of altered RBC deformability.

It is interesting to hypothesise regarding Leucodepletion.

The quality of blood storage should ideally be assessed using clinical markers of efficacy or *in vitro* measures that have been proven to be good surrogates for efficacy. Current quality parameters and accepted ranges may not be the best predictors of efficacy or guides to dosage. There is a need to identify those RBC properties that are important for RBC function

in vivo and, in principle, some of these should be used in the quality assurance of the product's efficacy.

10 Appendices.

10.1 Appendix 1: Adenosine triphosphate and 2,3 diphosphoglycerate assays.

10.1.1 Aim.

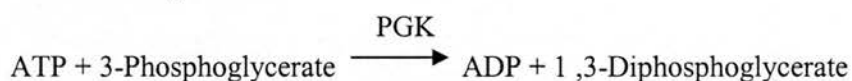
To determine the repeatability of the ATP and 2,3 DPG assays.

10.1.2 Methods: Adenosine triphosphate (ATP) assay.

SIGMA kit 366-A (Sigma-Aldrich Company Ltd, Gillingham, UK) was used. Ten healthy volunteers were recruited. A 2.5 mL EDTA blood sample was taken from each volunteer. Each sample was analysed twice for the ATP concentration. The Hb concentration of the samples was determined using a FBC analyzer (Sysmex KX21).

Principle

In the described procedure, the enzyme, phosphoglycerate phosphokinase (PGK), is used to catalyze the following reaction:



The enzyme glyceraldehyde phosphate dehydrogenase (GAPD) is also present in the reaction mixture to catalyze the following:



Where G3P is glyceraldehyde 3 phosphate and P_i is inorganic phosphate. By determining the decrease in absorbance at 340 nm that results when the reduced form of nicotinamide adenine dinucleotide (NADH) is oxidized to nicotinamide adenine dinucleotide (NAD), a measure of the amount of ATP originally present is obtained.

The assay.

1.0 mL blood was added to 1.0 mL ice-cold 12% trichloroacetic acid (TCA), mixed well and then left on ice for 5 minutes. The mixture was then centrifuged at 3000 rpm for 10 minutes

at 4°C. 0.5 mL of the supernatant, plus 1.0 mL phosphoglycerate (PGA) buffer and 1.5 mL H₂O was added to a 0.3mg NADH vial. The vial was then capped and inverted to dissolve the NADH. The vial's contents were then decanted into a disposable cuvette and the absorbance was read at 340 nm in a spectrophotometer using distilled water as reference (initial absorbance). Forty μL of the enzyme mixture GAPD/PGK was added to the cuvette and mixed by inversion. After approximately 10 minutes the absorbance was measured, again using distilled water as reference (final absorbance).

The ATP concentration was calculated using the following equations:

$$(\text{Initial} - \text{final absorbance}) \times 195 = \text{ATP } \mu\text{mol/dL}.$$

To express the ATP concentration as μmol/g Hb divide this value by the Hb concentration of the sample, determined using a FBC analyzer.

Data analysis.

The results are presented as mean (SD) unless otherwise stated. The within patient coefficient of variation was calculated using one way analysis of variance (statistical package SPSS 11.5.0)

10.1.3 Results.

The ATP results are shown in Table 10-1.

Table 10-1. The ATP concentration in blood samples taken from 10 healthy volunteers. The ATP concentration was measured twice for each sample.

Healthy volunteer	ATP concentration ($\mu\text{mol/g Hb}$)	
	1 st test	2 nd test
1	5.04	5.41
2	3.82	4.05
3	4.19	4.57
4	5.15	4.76
5	4.39	4.67
6	4.13	4.38
7	4.40	4.93
8	4.75	5.04
9	4.25	4.02
10	4.12	3.90

The mean ATP concentration was 4.5 (0.45) $\mu\text{mol/g Hb}$. The within-patient coefficient of variation was 5.2%.

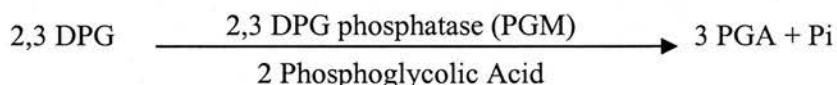
10.1.4 Methods: 2,3 DPG assay.

SIGMA kit 35-A was used.

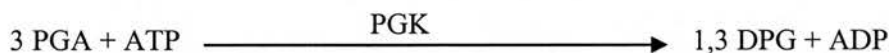
Principle

The three enzymatic reactions involved in the described procedure are as follows:

2,3 DPG is hydrolyzed to 3 PGA and P_i . The enzyme that catalyzes this reaction is present in purified preparations of PGM and is termed 2,3 DPG phosphatase. 2 Phosphoglycolic acid is needed as a stimulator for this reaction.



3 PGA reacts with ATP in the presence of PGK to form 1,3 diphosphoglycerate (1,3 DPG) and adenosine diphosphate (ADP).



1,3 DPG oxidizes NADH to NAD in the presence of GAPD and is reduced to glyceraldehyde 3 phosphate (G3P).



Measuring the decrease in absorbance at 340nm caused by the oxidation of NADH to NAD reflects the amount of 2,3 DPG originally present.

EDTA blood samples were obtained from 8 healthy volunteers. The Hb concentration of each sample was determined using a FBC analyzer (Sysmex KX21). The 2,3 DPG concentration of the sample was determined using SIGMA kit 35-A. The assay was performed 5 times on each sample.

The assay.

The following reagents were prepared:

Five mL triethanolamine buffer was added to the ATP vial. Five mL distilled water was added to the phosphoglycolic acid vial. Eight mL triethanolamine buffer was added to the nicotinamide adenine dinucleotide (NADH) vial. The vial was then capped and inverted several times to dissolve the NADH.

After preparation of the reagents 1 mL of the blood sample was added to 3 mL ice-cold 8% trichloroacetic acid (TCA) solution, the mixture was shaken vigorously for 10 seconds and then kept cold for 5 minutes. The mixture was centrifuged, 3000 rpm for 10 minutes at 4 °C, and then 0.25 mL supernatant was added to a cuvette with 2.5 mL solution from the NADH vial, 0.1 mL ATP solution. The cuvette was mixed by inversion and then 20 µL GAPD/PGK

enzyme mixture and 20 μL phosphoglycerate mutase (PGM) were added to the cuvette. Again, the cuvette was mixed by inversion. After 5 minutes the sample was read against water at 340nm in a spectrophotometer (initial absorbance). Then 0.1 mL phosphoglyceric acid was added to the cuvette. The cuvette was inverted and then left to stand for 30 minutes at room temperature to allow the reaction to go to completion. The cuvette was then read against water at 340 nm in a spectrophotometer (final absorbance).

The 2,3 DPG concentration was calculated using the following equation:

$$2,3 \text{ DPG } \mu\text{mol/mL} = [(\text{initial} - \text{final absorbance}) - 0.03] \times 7.7$$

Multiplied by 100 to give $\mu\text{mol/dL}$.

Divided by Hb g/dL to give 2,3-DPG $\mu\text{mol/gHb}$.

Data analysis.

The results are presented as mean (SD) unless otherwise stated. The within patient coefficient of variation was calculated using one way analysis of variance (statistical package SPSS 11.5.0)

10.1.5 Results

The 2,3 DPG concentration of the samples is shown in Table 10-2. The within-patient coefficient of variation was 8%.

Table 10-2. The 2,3 DPG concentration of the 10 healthy volunteers. The 2,3 DPG concentration of each sample was performed 5 times. The data are presented as mean (SD).

Healthy volunteer	2,3 DPG concentration ($\mu\text{mol/g Hb}$) Mean (SD)
1	11.4 (0.4)
2	10.3 (1.7)
3	12.6 (0.5)
4	14.9 (0.7)
5	13.2 (1.3)
6	16.1 (0.5)
7	11.1 (1.8)
8	11.0 (0.9)
9	12.6 (0.9)
10	13.3 (1.0)
Mean (SD)	12.7 (2.0)

10.2 Appendix 2: Extract from the “Guidelines for the Blood Transfusion Services in the UK” 6th edition 2002.¹⁴³ www.transfusionguidelines.org.uk/uk_guidelines

Table 10-3 Recommended tests for the evaluation of new red cell components.

New characteristic Parameter	New pack	Leuco- depletion	New centri- fugation / component extractor e.g. Optipress, Compomat, etc.	Novel AS / anti- coagulant	Apheresis OAS RBC	Apheresis OAS RBC with novel OAS / anti- coagulant	Gamma irradiated	Virus in- activated
Weight (g)	+	+	+	+	+	+	+	+
Unit vol (ml)	+	+	+	+	+	+	+	+
RBC $\times 10^{12}/U$	+	+	+	+	+	+	+	+
PCV%	+	+	+	+	+	+	+	+
Hb g/U	+	+	+	+	+	+	+	+
Reticulocytes $\times 10^{12}/U$					+			
WBC $\times 10^6/U$ (initial)	+	+	+	+	+	+	+	+
WBC $\times 10^6/U$ (post-L- D)		+						
Leucocyte subsets % (post L-D)		?						
Platelets $\times 10^9/U$	+	+	+	+	+	+	+	+
RBC recovery % (post L-D)		+						
Platelets recovery % (post L-D)		+	+	+	+	+	+	+
K ⁺ mmol/L	+	+	+	+	+	+	+	+
Haemolysis %	+	+	+	+	+	+	+	+
pH				+		+	+	+
FXIIa		+			?			+
Lactate mmol/L				+		+	+	+

New characteristic Parameter	New pack	Leuco- depletion	New centri- fugation / component extractor e.g. Optipress, Compomat, etc.	Novel AS / anti- coagulant	Apheresis OAS RBC	Apheresis OAS RBC with novel OAS / anti- coagulant	Gamma irradiated	Virus in- activated
Glucose mmol/L				+		+	+	+
ATP $\mu\text{mol/gHb}$				+		+	+	+
2-3,DPG $\mu\text{mol/gHb}$				+		+	+	+
Na ⁺ mmol/L				+		+	+	+
pCO ₂ kPa				+		+	+	+
pO ₂ kPa				+		+	+	+
PrP ^c and microvesicles		?		?		?		?
24 hour recovery %				?		?	?	?

Some components may need to be tested for a combination of parameters, e.g. apheresis red cells in a novel/experimental OAS that are also leucodepleted. In this case the sampling requirement includes that of a leucodepleted red cell component and that of an experimental OAS component

Key:

AS Additive solution

OAS Optimal additive solution

PrP Prion protein

L-D Leucodepleted

10.3 Appendix 3: Choice of buffer for RBC filterability assay.

10.3.1 Introduction.

Early experience with the St George's filtrometer in our laboratory gave very variable and inconsistent results (Table 10-4).

Table 10-4 Summary statistics of the red cell transit time (RCTT) data of "fresh" RBCs obtained from a healthy volunteer (HV) tested on two separate occasions. 10 RCTT measurements were performed on each occasion. The RBC suspensions were prepared as outlined in section 4.6.2, except that Dulbecco's phosphate buffered saline (PBS) without NaHCO_3 (Invitrogen, Renfrewshire, UK) was used instead of HEPES solution.

		RBCs from 1HV tested on 2 separate occasions	
		1st test (n=10)	2nd test (n=10)
RCTT (seconds)	Mean	12.33	19.08
	SD	3.02	4.23
	SEM	0.96	1.34
Within sample CV (%)		24.52	22.16

Light microscopy of the RBC suspension revealed a high proportion of echinocytes. It was suspected that this change in RBC morphology, and therefore the RCTT data, might be due to the high phosphate concentration of the PBS buffer. Although several previous studies have used PBS for filterability assays the actual composition of the PBS is often not specified.¹⁷² HEPES buffer has also been used.^{173;222}

Aim.

To compare the filterability of RBCs suspended in either HEPES or PBS buffer.

10.3.2 Methods.

A 20 mL venous blood sample was obtained from a healthy volunteer (SM). The sample was collected into EDTA anticoagulant. The sample was separated into two 10 mL aliquots. Each aliquot was then washed, leucoreduced using Imugard cotton wool (see Section 5.7.2) and diluted to an Hct of 0.07 using either PBS exclusively or HEPES buffer exclusively. RBC filterability was performed using the St George's filtrometer (see Section 5.7.3). Ten RCTT measurements were made on each RBC suspension.

10.3.3 Results

The RCTT data are shown in Figure 10-1 and Table 10-5.

Figure 10-1 Scatterplot of the red cell transit time (RCTT) of the RBCs prepared and suspended in either PBS or HEPES buffer.

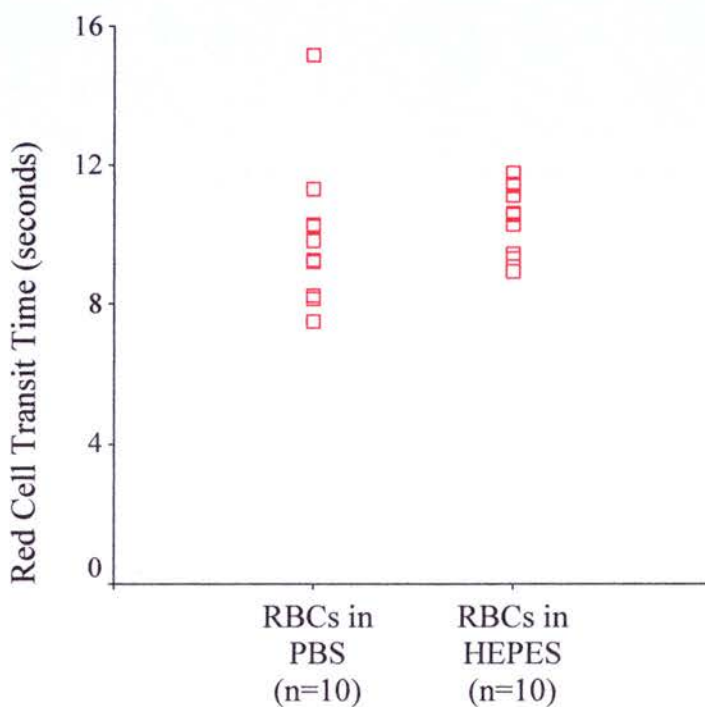


Table 10-5 Summary statistics of the RCTT data of RBCs suspended in either PBS or HEPES buffer.

10 RCTT measurements were performed with each RBC-buffer suspension.

		RBC suspension	
		in PBS buffer (n=10)	in HEPES buffer (n=10)
RCTT (seconds)	Mean	9.92	10.5
	SD	2.16	0.98
	95% CI of mean	8.56 to 11.28	9.88 to 11.12
	Within sample CV (%)	21.8	9.35

10.3.4 Discussion.

This study found that although the mean RCTTs of the two RBC-buffer suspensions were similar there was significantly greater variability with RBCs in PBS; the within-sample CV of RBCs in PBS was 21.8% compared to 9.35% for RBCs in HEPES buffer.

In conclusion, HEPES buffer is a suitable suspension medium for the assessment of RBC filterability using the St George's filtrometer.

10.4 Appendix 4: Validation of the differential agglutination technique.

10.4.1 Aim.

The 2,3 DPG assay was performed on multiple blood samples before and after differential agglutination to determine whether or not the separation technique affected the RBC 2,3 DPG concentration.

10.4.2 Methods.

Eighty mL of ("fresh") blood was obtained from a group O healthy volunteer. The blood was collected into 10 mL acid-citrate-dextrose anticoagulant.

A unit of stored group A RCC was provided by SNBTS. The unit had been collected and stored for 21 days as per SNBTS standard operating procedures. An aliquot of the stored blood was obtained from the pack via a sample site coupler.

The Hb and RBC 2,3 DPG concentration of the fresh and stored blood samples were determined (see Section 4.4.3). Each assay was performed 10 times on each sample.

The fresh RBC sample was then washed twice in phosphate buffered saline (PBS), following the second wash the sample was not re-suspended. Four mL of fresh packed RBCs were added to 7 mL of the stored blood; these proportions were chosen to achieve a similar ratio of O:A RBCs as obtained following a therapeutic transfusion of 2 units of RCC. The process was repeated to obtain 10 equivalent samples. A 100 μ L aliquot of each sample was fixed and labelled for flow cytometry (see Sections 7.5.2 & 7.5.3). The samples then underwent differential agglutination as described previously (see Section 7.4.5).

The amount of Hb recovered by this process was determined as described previously (see Section 7.4.5). The amount of Hb recovered by differential agglutination was expressed as a percentage of the group O Hb added to the original sample.

The 2,3 DPG concentration of the recovered RBCs was measured. A 100 μ L aliquot of the recovered group O RBCs was fixed, labelled and analysed by flow cytometry (see Sections 7.5.2, 7.5.3 & 7.5.4) to determine the degree of contamination with group A RBCs.

Data analysis.

The 2,3 DPG concentration of the group O blood samples before ("fresh") and after differential agglutination ("recovered") were compared using a paired t-test. The coefficient of variation (CV) of the 2,3 DPG measurements of the fresh and recovered RBCs were also reported.

10.4.3 Results.

The mean 2,3 DPG concentration of the fresh group O RBCs was 13.04 (SD 1.07) μ mol/g Hb (Table 10-6).

The 2,3 DPG concentration of the stored group A RBCs was undetectable.

The mean percentage of group A RBCs in the original 10 mixtures was 63 (SD 1.3)%.

A mean of 63% of the group O Hb was recovered by differential agglutination (Table 10-6).

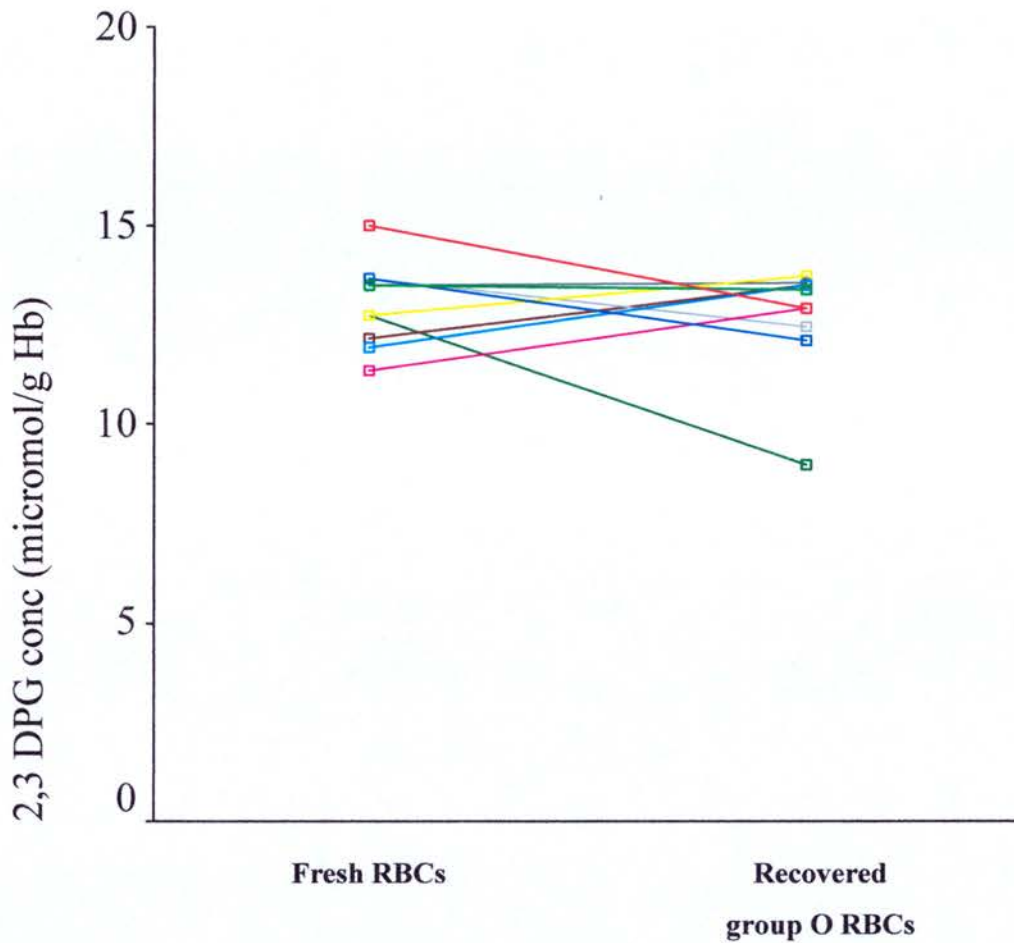
The degree of contamination of the recovered group O RBCs with group A RBCs was very low (<1%) in all of the samples (Table 10-6). The mean 2,3 DPG concentration of the recovered RBCs was 12.72 (SD 1.42) μ mol/g Hb.

There was no statistically significant difference between the 2,3 DPG concentration of the fresh and recovered group O RBCs ($p=0.586$) (Figure 10-2).

Table 10-6 The 2,3 DPG concentration of the fresh and recovered group O RBCs. The amount of group O Hb that was recovered by differential agglutination is expressed as a percentage of the amount that was originally present in the sample. The percentage of recovered RBCs that were A-antigen positive is reported as the degree of contamination.

RBC sample	2,3 DPG concentration ($\mu\text{mol/g Hb}$)		Recovery (%)	Degree of contamination
	Fresh group O RBCs	Recovered group O RBCs		(% of recovered group O RBCs that were group A)
1	15.03	12.95	73	0.16
2	13.50	13.42	47	0.17
3	13.68	12.14	75	0.07
4	11.34	12.92	72	0.1
5	11.97	13.53	71	0.11
6	12.73	13.73	78	0.09
7	13.69	12.46	69	0.13
8	13.53	13.54	59	0.11
9	12.18	13.52	76	0.11
10	12.73	8.96	19	0.11
Mean	13.04	12.72	64	
(SD)	(1.07)	(1.42)	(18)	
CV (%)	8.2	11.2		

Figure 10-2 The 2,3 DPG concentration of the fresh and recovered group O RBCs.



10.4.4 Discussion.

This study found that the technique of differential agglutination had no effect on RBC 2,3 DPG concentration.

Fresh, rather than stored, group O blood was used because it was expected that if differential agglutination affected the 2,3 DPG concentration it would cause it to decrease. 2,3 DPG depleted stored group A blood was chosen because it then meant that the two populations of RBCs had very different 2,3 DPG concentrations; high rates of contamination of the recovered group O RBCs with group A RBCs would result in a decrease in the measured 2,3 DPG concentration of the recovered RBCs.

The mean 2,3 DPG concentration of the fresh group O RBCs was within the normal range for our laboratory (mean 14.0 (SD 1.7) $\mu\text{mol/g Hb}$). The CV of 8.2% was acceptable. The mean 2,3 DPG concentration of the recovered group O RBCs was 12.72 $\mu\text{mol/g Hb}$ (CV 11.2%), which was not significantly different from the concentration reported in the fresh group O RBCs.

The 2,3 DPG concentration of sample number 10, following differential agglutination, clearly lies out with the 95% CI of the mean 2,3 DPG concentration of the recovered RBCs (11.7 to 13.6 $\mu\text{mol/g Hb}$). The reason for this is unclear; the amount of Hb recovered was much lower than expected whereas the rate of contamination with group A RBCs was typically very low (0.11%). The sample may have been haemolysed, it was certainly noted to have an abnormal appearance, but there is no corroborative evidence of this. Nevertheless, even including sample number 10 in the analysis, differential agglutination had no statistically significant effect on RBC 2,3 DPG concentration.

10.5 Appendix 5: In vitro validation of the anti-A indirect immunofluorescence labelling technique.

10.5.1 Aim.

To validate the anti-A indirect immunofluorescence labelling technique.

10.5.2 Methods.

A unit of time-expired group O RCC was provided by SNBTS. Twenty mL of fresh group A blood was obtained from a healthy volunteer. The RBCs were chemically fixed as described earlier (see Section 7.5.2). A haemocytometer was used to dilute the fixed group A RBCs with the fixed group O RBCs to obtain a series of red cell suspensions with final group A RBC concentrations ranging from 0-100%. An aliquot of each suspension was then labelled for flow cytometry using an indirect immunofluorescent technique directed against the A antigen as described earlier (see Section 7.5.3).

A-antigen positive (group A RBCs obtained from the healthy volunteer) and A-antigen negative (group O RBCs taken from the RCC) controls were prepared.

Data analysis.

The percentage of group A RBCs estimated by the haemocytometer and detected by the flow cytometer were compared using Pearson's correlation coefficient (r). The bias and limits of agreement were calculated using the Bland and Altman method.²²³

10.5.3 Results.

The percentage of group A RBCs detected by flow cytometry versus the percentage estimated by using the haemocytometer is shown in the Figure 10-3. There was a strong association between the two techniques ($r=1.00$, $p<0.01$). A Bland and Altman plot of the percentage of group A RBCs estimated with the haemocytometer and measured by flow cytometry is shown in Figure 10-4.

Figure 10-3 Plot of the percentage of group A RBCs measured by flow cytometry versus the percentage estimated with the haemocytometer.

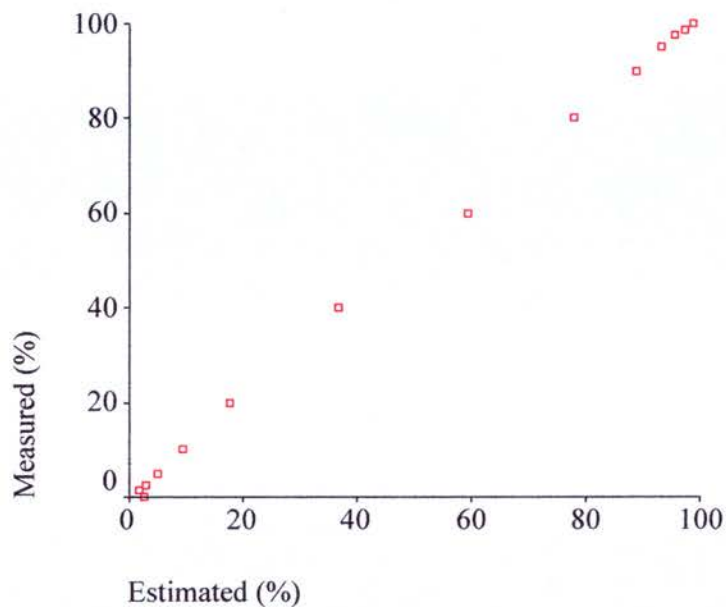
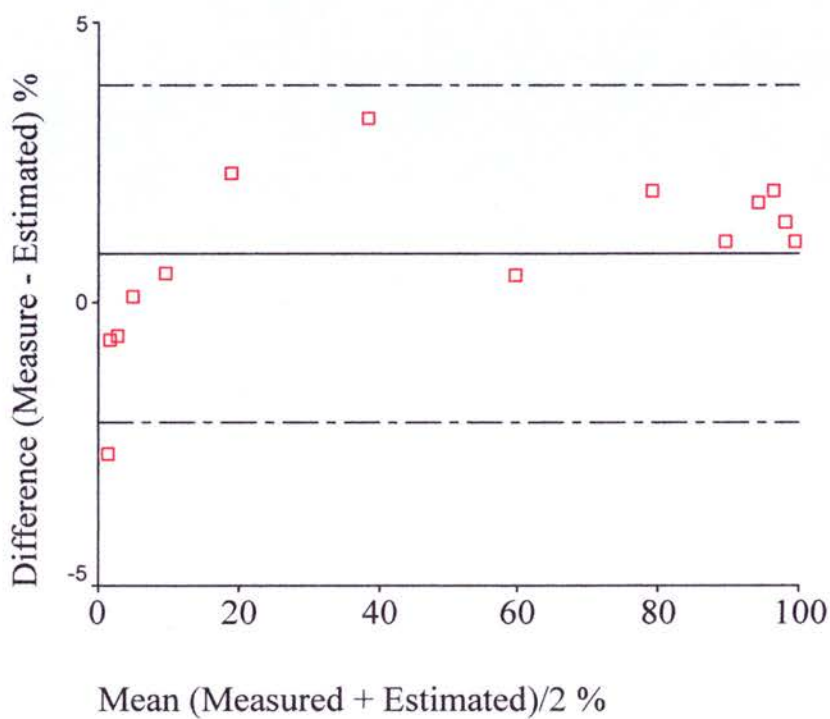


Figure 10-4. Bland and Altman plot of the percentage of group A RBCs measured by flow cytometry and the percentage estimated with the haemocytometer. LOA – 95% Limit of agreement.



10.5.4 Discussion.

The indirect immunofluorescence technique used here can accurately differentiate between group O and group A RBCs. The fixation technique does not appear to adversely affect the indirect immunofluorescence technique directed against the A antigen.

10.6 Appendix 6: Validation of RBC fixation method.

10.6.1 Introduction.

In Chapter 8 when the data from the first 4 critically ill patients were analysed it was noted that there was significant random variation of the proportion of group O RBCs (Figure 8-1). It was thought that this random variation might be due to error introduced by the fixation technique. The original fixation method (see Section 7.5.2) suggests centrifuging the blood sample and then aspirating a 100 μ L aliquot of packed RBCs from the RBC pellet. It was hypothesized that a density gradient within the RBC pellet may lead to uneven distribution of the autologous and stored allogeneic RBCs within the pellet. This was tested.

10.6.2 Aim.

To determine if sampling from a packed RBC pellet or from a RBC suspension influences the measurement error of the flow cytometry method.

10.6.3 Methods.

Two time-expired units of RCC were provided by SNBTS (one group O RCC and one group A RCC). Approximately 50 mL of blood was taken from each RCC pack using a sample site coupler. The RBC samples were washed with phosphate buffered saline. Aliquots of group A and group O RBCs were then added together to obtain two RBC suspensions with O:A ratios of approximately 1:2 and 2:1. Each of the RBC suspensions contained >5 g Hb; this was to minimise the effect of repeated sampling. The two RBC suspensions were centrifuged at 200 g for 5 minutes. Then, ten 100 μ L aliquots of packed RBCs were aspirated from the RBC pellet of each RBC mixture. The RBC mixtures were then re-suspended using a vortex mixer and ten 200 μ L aliquots of RBC suspension were taken. A total of 40 RBC samples were obtained, twenty 100 μ L aliquots of packed RBCs and twenty 200 μ L aliquots of suspended

RBCs. Each of the RBC samples was fixed, labelled and analysed by flow cytometry as described previously (see Sections 7.5.2, 7.5.3 and 7.5.4 respectively).

A-antigen positive (group A red cells) and A-antigen negative (group O red cells) controls were prepared.

Anti glycophorin A antibody was used to confirm the RBC gating.

Data analysis.

The within-sample coefficient of variation (CV) was used as an index of measurement error.

10.6.4 Results.

The proportion of group A RBCs detected in each sample is shown in Table 10-7. The mean, SD and within-sample CV are also shown in Table 10-7.

Table 10-7 Flow cytometry results showing the percentage of A-antigen positive RBCs in each of the samples taken from the pellet and suspension of RBC mixtures 1 and 2

Sample	Percentage of Group A RBCs (%)			
	Mixture 1		Mixture 2	
	Susp	Packed	Susp	Packed
1	62.5	58.9	30.7	39.8
2	62.8	64.5	33.1	43.2
3	68.4	71.8	35.6	32.5
4	64.0	62.5	35.1	41.2
5	65.4	64.9	35.4	34.6
6	65.4	60.6	35.1	40.4
7	66.0	66.2	35.0	33.4
8	67.1	72.0	36.9	31.2
9	67.0	65.0	29.3	34.6
10	66.1	64.9	37.0	30.2
Mean	65.5	65.1	34.3	37.3
SD	1.9	4.2	2.5	4.6
CV	2.9	6.5	7.4	12.4

10.6.5 Discussion.

This experiment found that the within-sample CV was greater when the RBC sample was aspirated from the RBC pellet rather than from the suspension. This may be due to uneven distribution of the two RBC populations within the RBC pellet. Such a density gradient may be even more marked when stored and fresh RBCs are mixed together, rather than stored and stored RBCs as in this study.

10.6.6 Recommendations:

Aliquots of RBCs for fixation should be aspirated from a suspension rather than a pellet of packed cells.

In addition, statistical theory states that a larger sample size will reduce sampling error (the difference between the measured value and the true value), therefore, in the context of this study, the mean of duplicate or triplicate samples should be more precise than single measurements.

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12.1 Papers:

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