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A Review of the Pathophysiology and Consequences of Red Cell Storage - Fresh Versus Stored Red Cells - Implication for Optimum Use of Scarce Allogenic Blood

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Abstract: About 90 million units of red blood are transfused worldwide yearly. Transfusion of red cell saves lives enabling complex haemorrhage prone surgeries and therapies to manage anaemia to be carried out. Challenges associated with the transfusion of allogenic blood including immunological and risk of transfusion transmissible infections. Red cells are stored at a temperature of 4 ± 2°C. At this storage temperature, it is ow possible to potentially store blood intended for transfusion for up to 42 days. However, due to red cell lesion-related challenges, stored red blood cells undergo a progressively degradation resulting in depletion of energy source to operate the physiological processes. This often affects the structural integrity of the red cell membrane, the deformability that allow it to pass through microcirculation to perfuse tissues and it fragility resulting in some level of haemolysis and the release of haemoglobin contained in its cytoplasm into the plasma. There had been an ongoing debate on whether it really matters in terms of optimum patient care what red cells transfusion a patient receives (fresh or older blood). Many have argued that the fresher the RBCs the better why others are of the opinion that there is no difference in patient outcomes between those transfused older and fresher RBCs. However, evidence seems to show that the transfusion of stored RBCs particularly those close to the end of the approved shelf life can potentially lead to morphologic changes that can potentially affect the ability of red cells to carry out the function of oxygen delivery. These changes include changes in cell shape from biconcave to spherical, increase in red cell adhesiveness to vascular endothelium, a decline in deformability and capillary flow, increased concentration of iron in the plasma due to release of free iron into the plasma due to haemolysis increasing the risk of nosocomial infections, thrombosis, vascular dysfunction, oxidative damage and associated challenge of organ failure, increased hospital stay and potential death. Transfusing fresher RBCs may benefit some patient cohorts, such as neonates, sickle cell disease, thalassaemic, septic and other severely ill patients particularly those in intensive care units while offering a limited or no benefit for the majority of other patients. Older RBCs might be given to certain patients' groups, such

as those with trauma having a major haemorrhage and extensive surgical procedures requiring large amounts of RBCs. However, many can also potentially argue that these patients may benefit most from fresher RBCs. Implementing and maintaining any policy of fresher RBCs have cost implication and may be associated with a significantly higher rate of outdating. It may also require major operational changes to the current practice of collection, storage and transfusion that can potentially affect the optimum use of scarce allogeneic blood particularly in developing countries where blood is scarce and available stock is inadequate to manage the transfusion needs of patients. There is the urgent need for the development of more optimum storage solution that can reduce the red cell lesion related challenges associated with cold storage of RBC. There is need for the shelf life of red cells to be determined and strictly regulated based on evidence on the basis of large and pragmatic clinical trials on the vitro haemolysis and in vivo recovery and survival. This will help maximize the suboptimal allogeneic stock of donor blood particularly in developing countries where there is difficulty meeting the increasing demand and transfusion needs of a vast number of patients.

Keywords: Pathophysiology, Consequences, Red Cell Storage, Fresh Blood, Stored Blood, Allogenic Blood, Developing Countries

1. Introduction

Red cell transfusion therapy is a common and effective treatment modality in the management of anaemia [1]. Today, blood collection and administration has become relative safe and efficient than ever before. However, during cold storage of blood, red cells undergo multiple red cell lesion associated metabolic and structural changes that can potentially compromise their deformability and by extension their functionality and viability following transfusion. The clinical relevance of these changes and the impact on transfused patient clinical outcomes has been a hotly debated topic triggering intense investigation and research [1].

Modern blood banking has evolved over the years and has made major remarkable achievements. Over the past 500 years, blood transfusion has progressed from vein-to-vein often fatal human transfusions (to both parties), to a highly efficient blood component therapy associated with the supply of a wide array of remarkably safe blood components (red cell concentrates, platelet concentrate, fresh frozen plasma, cryoprecipitate and other fractionated plasma products) to meet the therapeutic need of an expanding number of patients. Many important discoveries have contributed to this advance, one of which was the development of anticoagulant and preservative storage mediums in the 1940s that allowed red blood cells to be stored for prolonged periods while retaining much of their functionality and viability [1].

Modern anticoagulant and preservative mediums for the most part now potentially consist of various combinations of saline, glucose/dextrose, adenine, phosphate and mannitol [2, 3]. The main regulatory criteria that determines the maximum amount of time that red cells can be optimally stored (refrigerated at 4 ± 2°C) using these mediums include; the amount of haemolysis during storage (should ideally be <1% of the total haemoglobin in the unit) and how much of the transfused red cells that survives in the patient circulation for more than 24 hours (at least 75% of the red cells should survive for more than 24 hours post-transfusion) [4, 5]. These standards have not been substantially altered since the 1940s [6] and they are somewhat subjective and does not seem to consider whether transfusing fresher red cells improves patient outcomes better or have any clinical advantages over transfusing older red cells [1]. However, based on these standards, the allowable shelf life of stored red cells has increased from 21 days in the 1940s to 42 days or higher with the currently available anticoagulant-preservative mediums [2]. Over the past decade, the potentially harmful effects of storage on red cell functionality and viability have come under intense scrutiny raising questions about the clinical relevance of the storage lesion and the significance of currently approved optimum storage durations [2].

1.1. The Red Blood Cell

Red blood cells or erythrocytes are the most abundant cells in the blood stream and contains haemoglobin which plays a key role of transporting oxygen to the tissues in the body. Although haemoglobin can occur in a free state in some animals, it is however contained within the cytoplasm of the red cells in the human body. Any potential disruption of the red blood cells that potentially affects its shape, quantity, size, structure, physiology and by extension its life cycle can affect its ability to carry out its principal function of oxygen transport to tissues. Apart from carrying oxygen which is the main function of red blood cell, it also releases the enzyme carbonic anhydrase allowing water in the blood to carry carbon dioxide from the tissues back to the lungs to be expelled. It also plays a key role in optimizing the pH of blood by acting as an acid-base buffer [7].

Red cells are biconcave disc in shape. This facilitates its ability to permeate the microcirculation. A red blood cell is typically 6 to 8 micrometers in diameter (average diameter of 7. 8 um) with an average thickness of 2 micrometers. Although a red blood cell is typically wider than some micro capillaries, its flexibility however allows it to become distorted as it squeezes through microcirculation and then returns to its original shape [8]. The average male adult has about 5 million RBCs per cubic millimeter of blood, while the average female adult has about 4. 5 million RBCs per cubic milliliter of blood. This may vary depending on altitude and geographical location [9].

1.2. History

Jan Swammerdam, a Dutch biologist and microscopist was the first to observe and describe red cells in 1668. Antonie van Leeuwenhoek, another Dutch microscopist, was the first to report, in Philosophical Transactions of the Royal Society in 1675, a remarkable description of the unique features of human RBC. He stated, "when greatly disordered, the red cells appeared hard and rigid, but grew softer and more pliable as his health returned: whence he infers that in a healthy body they should be soft and flexible that they may be capable of passing through the capillary veins, arteries and micro circulation by easily changing their biconcave shape into ovals and also reassuming their former roundness when they come into vessels" This outstanding observation made more than 300 years ago, has remained proven and accurate. A brilliant review by [10] provides a comprehensive description of significance and recognitions for the discovery and description of red cells.

The non-nucleated erythrocyte is unique among human cells in that the membrane accounts for all of its varied antigenic, transport, and mechanical characteristics. The disc shape of the red cell grows from the multilobulated reticulocyte during 48 hours of maturation first in the bone marrow and then in the circulation [11]. A very important and unique feature of the disc shaped human red cell is its ability to undergo deformations during its repeated passage through the microvasculature throughout its 120-days life span [11-12].

Several studies in the past three decades on red cells from healthy people and from patients with various inherited red cell membrane disorders have showcased the molecular processes underlying normal and abnormal red cell membrane function [13].

1.3. Present View of Structural Organization of Normal Red Cell Membrane

The structural organization of the human red cell membrane allows it to undergo reversible deformations while maintaining its structural integrity during its 120-days lifespan in the human circulation. The red cell membrane is highly elastic and rapidly responds to applied fluid stresses. The red cell membrane envelope is composed of cholesterol and phospholipids. It is anchored to a 2-dimensional elastic network of skeletal proteins through fastening sites on cytoplasmic domains of transmembrane proteins entrenched in the lipid bilayer [14]. Direct interface of several skeletal proteins with the anionic phospholipids allow for additional attachments of the skeletal network to the lipid bilayer [15].

1.4. Red Cell Membrane Lipids

The lipid bilayer is composed of cholesterol and phospholipids [16]. Phosphatidylcholine and sphingomyelin are largely located in the outer monolayer, while most phosphatidylethanolamine and all phosphatidylserine (PS), together with the minor phosphoinositide constituents are restricted to the inner monolayer [17]. There are different types of phospholipid transport proteins that play a role in generating and maintaining phospholipid asymmetry [18]. There are several different membrane proteins play a key role in lipid transport activities in human red cells [11, 19]. Loss of lipid asymmetry has been shown to play a role in premature destruction of thalassemic and sickle red cells [20-24].

1.5. Red cell Membrane Proteins

Several transmembrane proteins describe the various blood group antigens. The membrane proteins show varied functions; transport proteins, adhesion proteins involved in interactions of red cells with other blood cells and endothelial cells and signaling receptors [11].

Membrane proteins with transport function include band 3 (anion transporter), aquaporin 1 (water transporter), Glut 1 (glucose and L-dehydroascorbic acid transporter), Kidd antigen protein, RhAG, Na^+-K^+-ATPase, Ca^{++} ATPase, Na^+-K^+-$2Cl^-$ cotransporter, Na^+-Cl^- co-transporter, Na^+-K^+ co-transporter, K^+-Cl^- co-transporter, and Gardos Channel. Membrane proteins with adhesive function include ICAM-4, which networks with integrins and Lu; the laminin-binding protein [25]. Of direct relevance to structural integrity of the membrane are two macromolecular complexes of membrane proteins (ankyrin-based and 4. 1R-based) [11]. Band 3 and RhAG link the bilayer to the membrane skeleton (through the collaboration of their cytoplasmic domains with ankyrin) and glycophorin C, XK, Rh and Duffy [20]. Recent studies have indicated that two other members of the spectrin-actin-protein 4. 1R junctional complex; adducin and dematin can also serve as linking proteins by relating with band 3 and Glut 1 respectively [26]. These membrane protein linkages with skeletal proteins may play a role in regulating cohesion between lipid bilayer and membrane skeleton and thus enable the red cell to maintain its favorable membrane surface area by preventing membrane vesiculation. In addition to its connecting function, band 3 also assembles various glycolytic enzymes; the presumptive CO_2 transporter and carbonic anhydrase into a macromolecular complex termed a "metabolon," which may play a key role in regulating red cell metabolism and ion and gas transport function [27].

1.6. Red Cell Skeletal Proteins

The principal protein constituents of the 2-dimensional spectrin-based membrane skeletal network are α- and β-spectrin, actin, protein 4. 1R, adducin, dematin, tropomyosin and tropomodulin [28]. A unique structural feature of the long filamentous spectrin is its large number of triple-helical repeats of 106 amino acids, 20 in α-spectrin and 16 in β-spectrin. These triple-helical bundles define a spectrin super family of proteins that includes dystrophin, actinin, and utrophin [20]. α- and β-spectrin form an antiparallel heterodimer through strong lateral collaboration between repeats 19 and 20 near the C-terminus of α-spectrin with repeats 1 and 2 near the N-terminus of β-spectrin [20]. The 36 triple-helical repeats of spectrin are organizationally

heterogeneous in terms of their thermal stability. Spectrin tetramer; the major structural component of the 2-dimensional skeletal network is formed by the lateral interaction of a solitary helix at the N-terminus of the α-chain from 1 dimer with 2 helices at the C-terminus of the β-chain from the other to create a stable triple-helical repeat [20]. While the spectrin dimer-dimer interaction was initially thought to be static, recent studies now shows that dissociation of spectrin tetramers can be induced by membrane deformation [29]. The other end of the 100 nm-long spectrin dimer forms a junctional complex with F-actin and protein 4. 1R. While actin interacts poorly with N-terminus of β-spectrin; the interaction is greatly enhanced by protein 4. 1R. The length of the actin filaments in the red cell membrane appears to be tightly regulated, probably by tropomyosin, and is made up of 14 to 16 actin monomers [29]. Adducin and tropomodulin cap actin filaments at reverse ends, while the function of the actin bundling protein dematin in the junctional complex has yet to be fully defined, the spectrin dimer-dimer interaction and the spectrin-actin-protein 4. 1R junctional complex are key regulators of membrane mechanical stability and play a critical role in preventing deformation-induced membrane fragmentation as the cell encounters high fluid shear stresses in circulation [20].

While much advancement has indeed been made in our understanding of the structural organization of the various lipid and protein components of the normal red cell membrane, the current models are far from all-inclusive and continue to evolve [11]. A recent study using state-of-the-art proteomic approaches has generated a comprehensive catalog of red cell proteins and has identified more than 300 proteins including 105 integral membrane proteins. The current membrane models account for fewer than 15% of these molecules [11].

1.7. Implications of the Structural Organization of Various Membrane Components for Normal Red Cell Function

In executing its primary function of oxygen delivery to the tissues, the red cell has to absorb continuous mechanical sentence throughout its lifetime without structural deterioration. Extensive biophysical studies have shown 3 constitutive features as the primary regulators of the ability of the red cell's capacity to undergo the necessary deformations. They include; the geometry of the cell; particularly cell surface area to volume ratio, the cytoplasmic viscosity determined by intracellular haemoglobin concentration and membrane deformability [11].

1.8. Red Cell Geometry

The normal red cells are biconcave and have a volume of 90 fL and surface area of 140 μm^2. It possesses an excess surface area of 40% compared with a sphere of the same volume. In the absence of excess surface area to volume ratio, the cell cannot deform for any deviation from the spherical state at constant volume implying an increase in surface area, which is prohibited by the lipid bilayer properties. Maintenance of membrane surface area is mediated by strong consistency between the bilayer and the membrane skeleton that prevents membrane vesiculation and by a mechanically stable spectrin-based membrane skeleton that prevents membrane disintegration. Maintenance of cell volume is mediated by various membrane-associated ion transporters. Surface area loss as a result of membrane vesiculation due to decreased membrane cohesion or cell fragmentation as a consequence of reduced membrane mechanical stability as well as increase in cell volume due to defective ion transporters can potentially compromise the ability of the cell to deform leading to its premature removal from circulation [11]. The determinant of normal membrane cohesion is the system of vertical linkages between bilayer and membrane skeleton, formed by the interactions of the cytoplasmic domains of various membrane proteins with the spectrin-based skeletal system [23]. Band 3 and RhAG provide such links by interrelating with ankyrin, which in turn binds to β-spectrin. Protein 4. 2 binds to both band 3 and ankyrin and can regulate the avidity of the interaction between band 3 and ankyrin. Glycophorin C, band 3, XK, Rh, and Duffy all bind to protein 4. 1R, the third member of the ternary junctional complex with β-spectrin and actin [23].

1.9. Cytoplasmic Viscosity

The ability of normal red cells to quickly change their shape in response to fluid shear stresses is determined by cytoplasmic viscosity, which is determined by intracellular haemoglobin concentration. Non-nucleated human red cells regulate their mean cell haemoglobin concentration within a very narrow range (30-35 g/dL) while their mean cell volumes range can vary widely (20-200 fL). While the mean cell haemoglobin concentration of normal human red cells is 33 g/Dl. The distribution of haemoglobin concentrations in individual red cells in whole blood ranges from 27 to 37 g/dL. The viscosity of haemoglobin solution increases sharply starting at 37 g/dL. While haemoglobin viscosity is only 5 centipoise (cp) at 27 g/dL increasing to 15 cp at 37 g/dL, it rises tersely to 45 cp at 40 g/dL, up to 170 cp at 45 g/dL and 650 cp at 50 g/dL [15]. By tightly regulating haemoglobin concentration within a narrow range, red cells minimize the cytoplasmic sticky indulgence during cell deformation. Increases in haemoglobin concentrations above 37 g/dL markedly decrease the rate at which the cell recovers its initial shape after both extensional and bending deformations [29]. Thus, the ability of the cell to accommodate rapidly to a narrow capillary in the microcirculation will be compromised by increased cytoplasmic viscosity, and with it, its efficacy in tissue oxygen delivery. It is worthy to note that cell dehydration and the resultant increase in cytoplasmic viscosity only minimally affect red cell survival [29].

1.9.1. Red Cell Membrane Deformability

The main feature of the normal red cell membrane is its high elasticity, which enables the cell to rapidly respond to applied fluid stresses in the circulation. Evidence has shown that a critical role of spectrin-based skeletal network in general and spectrin in particular in determining membrane elasticity. An important structural feature of the long filamentous spectrin dimer is the succession of 36 spectrin repeats, 20 in α-spectrin and 16 in β-spectrin, which behave in part as self-reliantly folding units. A recent study showed the thermal stabilities of the 36 individual repeats expressed in terms of the mid-point unfolding transition vary widely, ranging from 21 to 72°C [9]. It is anecdotal that unfolding of the least stable spectrin repeats might affect membrane elasticity. A recent elegant study, based on labeling by fluorophores of sterically shielded cysteines of spectrin in intact membranes in conjunction with quantitative mass spectrometry, demonstrated that such cysteines indeed became available to the reagent when the cell was mechanically stressed, and that these groups were located in repeats of low stability [20]. These findings support the concept that the unfolding and refolding of distinct spectrin repeats make a major contribution to the elasticity of the normal red cell membrane.

During senescence, normal red cells lose surface area and volume with little loss of haemoglobin and as a consequence the cell density increasingly increases during the red cell's 120-day life span. Recent reports have documented that after extensive fluid loss, normal red cells as well as sickle red cells lose their ability to maintain their cation homeostasis and cell volume increases [27]. It has been suggested that this cell population may represent the end-stage normal red cells destined to be removed from cell circulation. Other hypothesized mechanisms for removal of senescent normal red cells include phagocytosis of senescent cells by macrophages either through recognition of clustered band 3 or PS contact on the outer monolayer of this cell population [27]. Although it is difficult to allocate the relative role of each of the various documented cellular changes that eventually results in the removal of senescent red cells from circulation, it is likely that some or all of them play some role in the process [27].

1.9.2. Erythropoiesis

In mammals, conclusive erythropoiesis first occurs in the foetal liver with progenitor cells from the yolk sac [30]. Within the foetal liver and the adult bone marrow, haematopoietic cells are formed continuously from a small population of pluripotent stem cells that produce progenitors committed to one or a few haematopoietic lines. In the erythroid lineage, the earliest dedicated progenitors identified ex vivo are the slowly proliferating burst-forming unit-erythroid (BFU-E). Early BFU-E cells divide and further differentiate through the mature BFU-E stage into rapidly dividing colony-forming unit-erythroid (CFU-E) [31]. CFU-E progenitors divide 3 to 5 times over 2 to 3 days as they distinguish and undergo many significant changes, including a decrease in cell size, chromatin strengthening and haemoglobinization leading up to their enucleation and expulsion of other organelles [32].

The life span of normal human RBCs is 120 days. Under normal conditions approximately 1% of RBCs are synthesized each day but RBC production can increase considerably during times of acute or chronic stress, such as acute trauma or haemolysis. Delicate short-term control of erythropoiesis is regulated by the kidney-produced cytokine erythropoietin (Epo), which is made under hypoxic conditions and stimulates the terminal proliferation and differentiation of CFU-E progenitors [33]. BFU-E cells respond to many hormones in addition to EPO, including insulin like growth factor 1 (IGF-1), glucocorticoids (GCs) and IL-3 and IL-6. In cases of chronic erythroid stress such as haemolysis, the number of CFU-E progenitors is insufficient to produce the needed RBCs even under high EPO levels and the body responds by producing more of these progenitors from BFU-E [34]. It is not entirely known which cells in the foetal liver or adult bone marrow produce these and other regulatory cytokines or how they relate to regulate the division of BFU-E cells and control their self-renewal and their ability to develop into more mature CFU-E progenitors [35].

At each stage of RBC production, a significant number of intracellular signal transduction proteins and transcription factors are activated and interact with a group of DNA-binding and other transcription factors and chromatin modifiers and regulatory RNAs. RBC production is controlled at various differentiation stages: through cytokines, transcription factors, and cofactors, post-translational modifications of histones, and miRNAs [35].

1.9.3. Extracellular Signals Regulating Proliferation and Differentiation of CFU-E Progenitors

Erythropoietin has been shown to be a key factor governing erythropoiesis and its role in regulating the expansion, differentiation, apoptosis, and activation of erythroid specific genes is well known [33]. The first phase of CFU-E erythroid differentiation is highly EPO- dependent, whereas later stages does not seem to dependent on Epo [36]. Similarly, EPO receptors are lost as erythroid progenitors undergo terminal proliferation and differentiation [37].

The extracellular protein fibronectin has also been shown to be important in erythropoiesis [38]. Fibronectin and EPO regulate erythroid proliferation in temporally distinct steps. A 2-phase model for growth factor and extracellular matrix regulation of erythropoiesis, has been suggested with an early EPO -dependent, integrin-independent phase followed by an EPO -independent, $\alpha_4\beta_1$ integrin-dependent phase [35].

Binding of EPO to EPO receptors (EpoRs) on the surface of erythroid progenitors' triggers the activation of numerous intracellular signal transduction pathways including the signal transducer and activator of transcription 5 (Stat 5), phosphoinositide-3 kinase/Akt, and Shc/Ras/mitogen-activated kinase (MAPK) pathways. Elimination of either of

the first 2 pathways leads to significant apoptosis of early progenitors and abridged output of erythrocytes [33]. In contrast, blocking the Ras/ MAPK pathway has only indirect effects on terminal erythropoiesis [39].

1.9.4. Life Cycle of Red Blood Cells

Red blood cells are produced from the haemopoietic stem cells in the bone marrow. They are known as erythtropoietic bone marrow cells and are partially differentiated. During red blood manufacture, these cells go through various phases of development until the mature red blood cell can be released into the bloodstream. The final stage of maturation requires two very vital vitamins – vitamin B12 and folic acid. This process of developing from erythropoietic bone marrow cells to mature red blood cells takes about 7 days [26].

The trigger for the production of red blood cells is hypoxia (low oxygen state). However, hypoxia alone will not be sufficient to trigger the production of new red blood cells unless there is adequate amount of the hormone erythropoietin in the bloodstream. Erythropoietin is primarily produced by the kidneys. The life span of a red blood cell is approximately 120 days but they may be removed out of circulation at any time if they are severely damaged, coated with antibody and non-functional. Most of the red blood cells self-destruct rather than being actively removed from the circulation and destroyed. The principal site where this occurs is in the spleen [26].

Once a red blood cell ruptures, the haemoglobin contained in the cytoplasm is released into the circulation for processing. This is primarily done by the Kupffer cells of the liver and macrophages in the spleen and bone marrow. These macrophages release the released iron which bound to and transported transferrin to the bone marrow where it can be reused for the production of new red blood cells. The remaining porphyrin portion of the haemoglobin molecule is further converted to bilirubin by the macrophages. The liver cells (hepatocytes) take up the bilirubin, conjugate it and release it in the bile [26].

1.9.5. Red Blood Cell Additive Solutions and Storage Condition

Red blood cell (RBC) transfusion is a vital life-saving treatment for the management of severe anaemia caused by disease or chemotherapy, haemorrhage due to trauma or major surgery. For several decades, RBC components, have been prepared as red cell concentrates suspended in nutrient additive solution which preserves and extends the shelf-life of the RBC component, allowing for up to 6–7 weeks of effective cold storage at 4± 2°C [40]. However, during storage RBCs undergo a multifaceted and progressive accumulation of physicochemical changes, collectively known as RBC storage lesion [41]. Recent clinical studies have identified RBC transfusion as a sovereign risk factor responsible for increased morbidities and mortalities in some groups of patients; including trauma, cardiac surgery and the critically-ill. Also, some of these reports have identified that older stored RBCs are more strongly implicated in poorer patients' outcomes compared to fresher RBCs [42]. In order to address these challenges, there is renewed desire in the scientific community to better understand the RBC storage lesion and to find ways to obviate the deleterious effects of storage, to facilitate the improvement of the quality, efficacy and safety of RBC components for all patients that require red cell transfusion [43].

1.9.6. RBC Additive Solutions - Old and New

The first RBC additive solution to come on stream was the saline-adenine-glucose (SAG) which was developed by European researchers in the late 1970s. Eleven years later, in 1981, the same researchers included mannitol to help protect the RBC membrane and reduce the incidence haemolysis enabling cold storage of red cells for up to 6 weeks at a haematocrit of approximately 55 to 60 percent [44]. This improved SAG formulation was named SAGM and up till date, SAGM is the most widely used RBC additive solution. However, in some countries SAGM red cell concentrate continued to be stored for only 5 weeks despite the fact that evidence has shown that SAGM is approved for 6 weeks cold storage of red cells [43]. SAGM has not yet been licensed by the Food and Drug Administration (FDA), and hence is not used in the USA. Other additive solutions, which are all essentially variations of SAG/SAGM, have since been developed and commercialized. They include; AS-1, AS-3, AS-5, MAP and PAGGSM [45]. These solutions seem to provide better preservation of RBCs compared to RBCs stored in SAGM, such as reduced risk of haemolysis and reduced shedding of microparticles from the RBCs. However, the specific biological mechanisms that are modulated in RBCs stored in the variant SAG/SAGM solutions have not been exactly identified [40].

All of the presently licensed RBC additive solutions have an acidic pH (5. 6–5. 8), which is well below the normal physiological pH of 7. 3 of venous blood. The reason why acidic additive solutions and anticoagulants are used is because it is easier to heat-sterilize a glucose-containing solution at an acidic pH. At physiological and alkaline pH, there is the tendency for glucose to caramelize during heat-sterilization [43]. It has been shown that RBCs have adequate buffering capacity to adjust the pH to nearer the physiological levels during the first few days of storage in an acidic environment. However, the buffering capacity of the RBCs is quickly exhausted due to the generation of lactic acid by the RBCs via the anaerobic glycolytic pathway [43]. Consequently, the extracellular and intracellular pH of RBCs gradually becomes more acidic during storage, reaching a pH of 6. 5 after 6 weeks of storage in acidic additive solutions [43].

An acidic intracellular environment alters enzymes activities and biochemical pathways. For RBCs, increased intracellular acidity interferes with the generation of adenosine 5′-triphosphate (ATP) the source of energy to drive the metabolic process and 2, 3-diphosphoglycerate (2, 3-DPG) which are crucial for the maintenance of the deformability, red cell survival and function in delivering oxygen [45]. During storage of RBCs in acidic additive

solutions, the intracellular concentration of ATP and 2, 3-DPG decline. Over the past two decades, extensive research into the development of new additive solutions has focused on ways to maintain higher levels of intracellular levels of ATP and 2,3-DPG during storage of RBC components [40]. The main driving force for this research had come from the military, who were keen to develop ways to store red cells for longer than 6 weeks to facilitate their inventory control management of blood component supply to battlefield hospitals and remote areas where regular shipments of fresh blood supplies are often not guaranteed [43]. However, longer storage time does not seem to be key to the civilian blood services. With the current concerns about poor outcome observed with the transfusion of older blood in certain patients' groups, there seem no motivation to extend the shelf-life of RBC components [46]. However, there should be a drive by the civilian blood services and the general medical community to support new technologies aimed at developing new RBC additive solutions that offer further enhancement to the quality and efficacy of RBC components that will benefit our customers and patients [43]. This particularly vital for developing countries that face a daunting challenge of supplying adequate and safe supply to meet the blood transfusion needs of their patients. Being able to effective store blood for longer than 6 weeks will reduce the risk of wastage due to outdating and will allow developing countries better manage their allogeneic blood stock.

Of potential interest are the alkaline, chloride-free hypotonic solutions which have been the focus of research by several scientists in the last 2 decades. The chloride-free preparations rely on chloride shift to establish a Donnan equilibrium between the charged ions in the intracellular and extracellular medium [47]. Consequently, in a chloride-free medium, intracellular chloride will leave the cell and in the absence of any other diffusible anion, hydroxide will enter the cell and raise the intracellular pH. The alkaline storage solutions contain glucose and thus have to be sterilized as 2 separate solutions to prevent glucose caramelization. This can potentially be achieved by having one pack contains the alkaline salts and the other pack contains the glucose solution. Both solutions can be combined at the time of RBC component manufacture [43]. After widespread testing of many variants, experimental preparations have been devised that appear to preserve RBC quality better than the conventional additive solutions, giving at least 2 weeks extra advantage to RBCs stored in these new generation additive solutions [43].

1.9.7. Storage Research - Expect the Unexpected

Regardless of the fact that RBC have been a favorite experimental model for cellular biologists and biochemists, RBC storage research has continually shown that there is still paucity of information on fundamental biology about RBCs. The intricacy of the inter-relationship between RBC biochemistry, cytoskeletal structure and membrane properties have made it problematic particularly in predicting how RBCs will respond to different storage conditions. Exposure of RBCs to non-physiological storage environments have pin pointed the existence of formerly unknown biochemical mechanisms in RBCs such as apoptotic-like processes, ion and osmotic channels that behave differently than expected, exposure of new or transformed receptors possibly due to oxidative and/or protease/glycosidase events or altered senescence [43].

Expect the unexpected seems a slogan that researchers working in the field of RBC storage have had to come to terms with. Evidence from a recent study showed that RBCs stored in an alkaline, chloride-free additive solution had higher intracellular concentrations of ATP and 2, 3-DPG as expected. However, the unexpectedly occurred as the intracellular pH became acidic within the first week of storage [47]. Hence, the premise that the so-called chloride shift operates by optimizing the intracellular pH of RBCs may need to be re-examined [47]. Also, an unexpected response was observed which seem to indicate that RBCs stored in experimental hypotonic alkaline, chloride-free additive solution appeared to dehydrate. This may be an indication that there is ion fluidity into and out of the RBCs stored under non-physiological storage conditions. This may be responsible for the altered behavior of the ion channels and co-transporters contrary to that which is currently known. It is imperative that much is still to be learned about the effects of storage conditions on RBCs [43].

1.9.8. Challenges for the Introduction of new RBC Storage Systems

Cost benefit analysis seems to justify the additional processing costs that blood services will need to incur by the introduction of new generation RBC additive solutions despite the inconvenience of implementing adjustments to their processing procedures [43]. The greater challenge that seems to have hindered the advancement of this field is the huge financial load and risk for manufacturers of blood collection systems to obtain licenses and to bring these new RBC storage systems to a market particularly with the characteristically associated low profit margins [43].

The huge monetary burden to technology developers of these new RBC storage systems is largely due to the bureaucratic regulatory requirements particularly those mandated by the FDA. In addition to requiring *in- vitro* data, the FDA often requires *in- vivo* data on the 24 hours post-transfusion recovery of transfused donor RBCs in the circulation of the recipient [43]. Lately, the FDA seem to have tightened and increased the assessment and acceptance criteria making it hypothetically more difficult and expensive to develop and bring new RBC storage systems to the market. Though the regulatory agencies deserve commendation for focusing on the safety of new therapies and devices for use on patients, there are however concerns that these regulatory requirements particularly for RBC storage systems seem to have become unnecessary excessive and hampering advancement in this area [5].

An additional important challenge for obtaining licensure of new RBC storage systems is the inherent blood donor-related variability in stored RBC quality. Evidence have shown that RBCs from some donors do not store well, compared to other particularly because there are higher levels of haemolysis at RBC component expiry in some donor units compared to others despite being collected at the same time and stored under similar circumstances [4]. There is also the challenge of variation and poor *in vivo* 24-hour recovery data [48]. The role of donor- specific and poorer quality of some stored RBC components was established in a recent paired cross-over study designed to compare manual and automated whole blood processing methods. Technology developers seems reluctant to take on the risk of a random poor-quality RBC component jeopardizing the success of licensure tests and clinical trials of their new blood storage systems considering their huge financial investment [43].

There are donor-specific factors that potentially contribute to the storage quality of RBC components which are yet to be identified [43]. There seems to be genetic, undiagnosed and sub-clinical medical conditions, donor's health, haematological status and donor lifestyle factors that are all likely to be involved [43]. RBCs from some donors may be more prone to oxidative and/or mechanical stresses encountered during *in- vitro* processing and storage. Evidence exist which seem to suggest that RBCs from young female donors are more resilient to mechanical stress than RBCs from male donors and post-menopausal women [49]. This may relate to hormonal factors of the female menstrual cycle. Also, several lifestyle factors including exercise, diet, alcohol consumption and smoking among others may contribute to RBC susceptibility to oxidative and/or mechanical stresses [50]

1.9.9. Red Blood Cell Shelf Life in Current Practice

For a significant part of the 20th century, the main aim of red cell preservative solution research was to extend the shelf life of red cells in order to improve the inventory control management of blood products and by so doing reduce the challenge of outdating [51]. The shelf life of red cells is determined and stringently regulated not on the basis of clinical studies only but also based on in- vitro haemolysis and in- vivo radiochromium label recovery and survival studies [48]. The tolerable limits were formerly derived from expert opinion and not necessarily through correlation with clinical outcome [5]. Most blood providers extended the RBC shelf life of red cells from 21 to 28 days with the addition of phosphate to the solution and to 35 days with the addition of adenine and to further 42 days when additive solutions became available widely used [52]. The lengthiest shelf life currently in clinical use is 49 days [53]. A new additive solution has lately (Conformite Europeenne; CE) been certified for 56 days cold storage in Europe and 42-days storage in the United States of America by the Food and Drug Administration (FDA) [40]. Some blood transfusion services worldwide have chosen to voluntarily retain 35 or 42 days and waive the opportunity to increase shelf life, despite national regulations allowing the extension of the shelf life to 42 or 49 days. Japan for example has chosen to restrict the 42-days red cell storage to a 21-days shelf life since 1995. The shorted storage period has not only reduced the risk of septic reactions from slow-growing bacteria, it has also permitted the universal irradiation of RBC to prevent transfusion-associated graft-versus-host disease (GvHD) [54].

Some other national blood systems have reduced the RBC storage interval in their expectation that the shortened shelf-life improves RBC quality. The United Kingdom limits RBC shelf life to 35 days by UK Blood Transfusion and Tissue Transplantation Services (2013) policy, notwithstanding the law [55] and scientific evidence that the use of additive solutions deemed stored blood suitable for 42 days. The Netherlands chose to apply 35-days storage even with the availability of additive solutions particularly because inventory control management of blood product is satisfactory with associated shorter outdate and discard rates remain <2%. Some other health care systems have established a policy of discarding RBCs, once storage has reached the last 7 days of the longest acceptable shelf life for their additive solutions. Large regions and entire nations have resolved that a 35-days shelf life is safe and cost-effective for the provision of RBC support for their patients [56].

Table 1. *Additive solution for RBC storage times currently approved and applied [56].*

Additives solution	Days approved	Days applied in clinical service
CPD (citrate-phosphate-dextrose)	21	21
CPDA-1 (citrate-phosphate-dextrose-adenine	35	35
MAP (mannitol-adenine-phosphate	42	2
SAGM (saline-adenine-glucose-mannitol	42	35
AS-3 (saline-adenine-glucose-citrate-phosphate	42	42
PAGGS-M (phosphate-adenine-glucose-guanosin-saline-mannitol	49	49
PAGGS-M (phosphate-adenine-glucose-guanosin-saline-mannitol	49	42

2. Red Blood Cell Concentrate Preparation

Blood transfusion can be a lifesaving procedure, but it associated with potential risks including infectious and non-infectious complications. There is debate in the medical community concerning the appropriate use of blood and blood products. Clinical trials investigating the use of blood seem to recommend that waiting to transfuse at lower haemoglobin levels is beneficial particularly because blood

transfusion is not an entirely safe process [57].

Red blood cell transfusions are used to manage haemorrhage and to enhance oxygen delivery to tissues. Transfusion of red blood cells should be based ideally be based on the patient's clinical condition. Indications for transfusion include symptomatic anaemia (causing shortness of breath, congestive heart failure, dizziness and decreased exercise tolerance), acute sickle cell crisis, acute blood loss of more than 30 percent of blood volume and other situations where red cell transfusion is clinically indicated [58].

2.1. Preparation from Whole Blood

Packed red blood cells (RBCs) are manufactured from whole blood by removing roughly 250 mL of plasma [58]. One unit of packed RBCs should potentially optimize the levels of haemoglobin by 1 g/dL (10 g/ L) and haematocrit by 3 percent. In most areas, packed RBC units are universally filtered to ensure leucodepletion of blood before storage. This has been shown to limits the risk of febrile non-haemolytic transfusion reactions (FNHTRs) and make blood intended for transfusion cytomegalovirus safe particularly to meet the need of CMV recipient and patients in whom cytomegalovirus negative units are indicated (neonates, pregnant women and immune-compromised patients such as HIV patients) [59].

Several RBC preparation and storage conditions have been studied to improve RBC survival in the storage container, as in- vitro changes are key to predicting clinical outcomes [5]. Within the past decade, RBC preparation and storage solutions have gone through different modification [60]. Though the approach has become generally standardized, several small technical variations exist that seem to have added to the inherent complexity of the optimum storage of RBC blood product [4]. It is however becoming increasingly clear that a biological product from human volunteer blood donors with associated variations, it may be extremely difficult to standardize the source.

Unlike small molecule drugs, each donor RBC unit is considered a ‘lot’ or a ‘batch. ’ A considerable donor-to-donor variability in RBC storage, haemolysis and survival has been documented since the 1960s with non-normal distribution, in that the RBCs of about two-thirds of the donors’ store better than the mean; there are donors whose RBC store remarkably well [5, 48]. There are currently no biomarkers to predict suboptimal storage and to identify donor units that don’t store well. It will be enormously valuable if the scientific community can develop biomarkers required to make this inference [61].

Whole blood is characteristically collected into a sterile plastic bag and anticoagulated with a well-defined concentration of citrate solution. The packed RBC are then transferred into a second bag with an additive solution, for which SAGM (sodium, adenine, glucose, mannitol, 376 mOsm/l) is frequently used and accepted for a 42-days RBC shelf life [40]. Approval of additive solutions depends on in-vivo RBC recovery and survival as well as on measurement of analytes that are known to be critical for RBC survival. The most important RBC analytes are adenosine 5′-triphosphate (ATP) concentration and 2, 3-DPG recovery rate [48]. RBCs are controlled by regulations that vary from one country to the other. In the United Kingdom for example, RBCs are controlled by the Drug Acts and Biological Medicine Regulations [55] while in the US by, the FDA policy and regulations [62]. Generally accepted RBC recovery and haemolysis rates after 12 weeks storage may be realized with non-standard solutions, which are yet to be approved [40].

Leucodepletion during RBC preparation is evidenced - based best practice in Europe and Canada, but not standard developing countries. RBC preparations differ in many regards that can be easily delineated in -vitro and may be of clinical relevance. Irradiation, which is used for specific indications in some countries like the UK or universally in others such as Japan is well documented to cause some level of damage to the RBC membrane. The plasticizer diethylhexyl phthalate (DEHP) which are widely used for RBCs has been shown to be associated with leaches from the polyvinyl chloride (PVC) bags. This has been shown to affect the RBC membrane stability and negatively affect membrane loss by microvesiculation beyond 3 weeks storage [40]. Although plasticizer is a critical parameter for approval of new blood container devices and are known to reduces haemolysis [63], possible DEHP toxicity remains a challenge particularly for premature infants [64].

The overall quality of a RBC concentrate may potentially improve during the first few days of storage. Bacterial contamination during the donation process may be cleared within the first few hours of storage of whole blood or RBC fraction, before the leucocytes are removed. Storage at 4°C ± 2°C for several days may reduce the risk of spirochetes, some cell-associated viruses and mononuclear cells that can potentially cause GvHD [65]. While no claim has been made that RBC, quality improves during storage beyond the first few days, a significant in- vitro evidence seems to implicate refrigerated storage as increasingly responsible for the progressive degradation in the quality of the RBC [66-67].

2.2. Preparation by Apheresis

Apheresis is a procedure where required single or more than one component of whole blood is collected and the rest of blood components are returned back to the donor. The principle upon which most apheresis equipment are based is either by centrifugation (different specific gravity) or by filtration (different size) [68]. The most commonly used apheresis equipment is based on the centrifugation principle and can also guarantee the production of leucodepleted products. In this method, fixed quantity of whole blood is collected in a bolus called as Extracorporeal Volume (ECV) and the required blood component (red blood cell, platelets or peripheral blood stem cells) is separated and collected in the collection bag and the other components that are not required are returned back to the donor. Centrifugation apheresis equipment is classified as either intermittent or continuous [69].

The *Intermittent* equipment uses single venous access for both collection and return. One cycle consists of one ECV whole blood collection in kit bowl, centrifugation of bowl to separate components, collection of required component (for example red blood cells) in a collection bag and finally the other constituents like leucocytes and plasma are returned to the donor. This cycle is repeated until therapeutic dose of the component is achieved [69]. In the continuous working equipment, two simultaneous venous access are established: One for the collection of the desired blood component and the other for the return. The collection, centrifugation, desired blood component collection and the unwanted component return occur continuously and simultaneously. Each type has its own advantages and limitations. The eventual goal of the procedure is not to exceed ECV collection more than 15% of total blood volume (TBV). At any point ECV should not reduce beyond 20% of TBV and the final product should not exceed 15% ECV of TBV to avoid hypovolemia [69].

2.3. Pathophysiological Changes of Red Cell During Storage

The main challenge of red cell storage is to maintain the functionality and viability of red cells throughout the approved shelf life or storage period of the red cells [41]. The key challenge that is common to modern storage mediums, however, is that red cell functionality and viability are progressively diminished during storage by three interconnected mechanisms: altered red cell metabolism; increased oxidative stress; and membrane damage [1].

2.4. Altered RBC Metabolism

The human red blood cells meet their metabolic needs by oxidative phosphorylation of glucose since that are non-nucleated and lack mitochondria. It therefore has to rely on anaerobic glycolysis to generate metabolites, primarily adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG), and reduced nicotinamide adenine dinucleotide (NADH) [70]. Adenosine triphosphate is the energy sources the numerous biochemical reactions in the red cells. Also, the 2, 3-DPG regulates the affinity of haemoglobin to oxygen, and NADH is an important co-factor that reverses the spontaneous oxidation of oxyhaemoglobin to methaemoglobin within the red cell [71].

Maintenance of the optimum storage temperature of 4 ± 2°C helps to maintain red cell function and viability by reducing the rate of the metabolic process. Each one degree drop in storage temperature can potentially result in about a 10% decrease in red cell metabolism [72]. Evidence has shown that at 4°C the metabolic rate is ten times lower than at 25°c [73]. The metabolic process does not come to stop when red cells are stored. This is the reason why glucose or dextrose are added to red cell storage mediums to enable the red cells to continue glycolysis and produce enough ATP, 2, 3-DPG and NADH required to drive the metabolic process required for the maintenance of red cell function and viability during cold storage. However, the continuing glycolysis during cold storage can potentially lead to the accumulation of the primary by-product of glycolysis including lactic acid which is deposited in the supernatant plasma [70]. The generation of lactic acid reduces the pH (blood become acidic). The resulting acidosis can potentially inhibit glycolysis through a negative feedback process resulting in a progressive reduction in ATP, 2, 3-DPG and NADH levels [74]. By the time blood has been stored for six weeks the amount of lactate in the plasma has increased by several folds resulting in a drop in pH to potentially less than 6. 5. The ATP and NADH contents are substantially reduced, and 2,3-DPG content is often depleted [75]. Reduced ATP levels can affect red cell metabolic activities including glycolysis itself (ATP plays an important role in the initial steps of glycolysis) as well as the production of cytosolic antioxidants (reduction in antioxidant defenses) and maintenance of red cell membrane integrity (reduction in red cell deformability and alterations in their disc shape) [76]. There seems a direct relationship between reduced ATP levels and red cell viability [77]. The agent 2, 3-DPG play a key role in the ability of the red cell to carry out its primary function of oxygen transport. The depletion of 2, 3-DPG reduces perfusion of tissues. However, evidence has shown that the levels are rapidly replenished 48-72 hours after transfusion [70] (Greer, 2014). Another major challenge associated with storage lesion is the depletion of reduced NADH, which can potentially affect the conversion of methaemoglobin back to oxyhaemoglobin inside the red cell, thus provoking oxidative stress [78].

Another outcome of cold-storage of red cells is that it affects the exchange of sodium and potassium across the red cell membrane by the sodium/potassium adenosine triphosphatase (Na+/K+ ATPase) pump [79]. Under normal situations, the concentration of potassium and sodium within the red cell are preserved at approximately 90 mM and 5 mM, respectively. However, outside, they are roughly 5 and 140 mM, respectively [80]. By the time red cells have been stored for six weeks, the potassium content would have fallen by about 40% and the sodium content would have increased by about 300% [79]. In the same manner, the potassium content in the supernatant plasma of the stored unit of blood is significantly increased. This can potentially to hyperkalaemia after transfusion [74]. The resultant increased intracellular sodium content can negatively affect cell volume and shape. This may be responsible for the increased mean corpuscular volume (MCV) of stored red cells after 3 weeks of storage [80]. This effect is more evident in older red cells thus affecting their deformability and extension their viability [79].

2.5. Increased Red Cell Oxidative Stress

For haemoglobin to be able to reversibly bind oxygen (oxyhaemoglobin ↔deoxyhaemoglobin) within the red cell, the heme-iron must be maintained in its reduced ferrous (Fe^{2+}) state [1]. Under normal circumstances, a small amount of oxyhaemoglobin can undergo spontaneous oxidation

leading to the production of methaemoglobin (oxidized ferric iron; Fe^{3+} and cannot bind oxygen) and reactive oxygen species [81]. Methaemoglobin is characteristically unstable and is denatured into globin and haemin (ferric or oxidized haeme) [70]. Some of which may in turn be broken down by erythroid haeme-oxygenase-1 to release its iron [82]. Free haemin and iron, in combination with reactive oxygen species, can produce highly harmful hydroxyl radicals that can cause oxidative injury to membrane lipids and proteins [83]. Under normal circumstances, red cells are protected against oxidative stress injury because the rate of spontaneous oxidation of haemoglobin is slow [84], the NADH-dependent cytochrome-b5 reductase (CYTb5) reduces methaemoglobin back into oxyhaemoglobin, and cytosolic antioxidants (primarily reduced glutathione or GSH) and membrane anti-oxidants (primarily ascorbic acid or vitamin C) counterbalance the generated reactive oxygen species [85].

All of these protective mechanisms are impaired during storage [1]. Spontaneous oxidation of haemoglobin to methaemoglobin increases under conditions of increased oxygen partial pressure and acidosis [86], both of which are present during storage [87]. The rate of oxidation of haemoglobin to methaemoglobin is substantially increased during storage [88]. Moreover, due to the metabolic changes, levels of NADH, GSH and ascorbic acid are significantly reduced during this period [89]. Increased spontaneous oxidation of haemoglobin in the setting of reduced antioxidant defenses exposes red cells to increased oxidative stress, which is the principal cause of red cell membrane damage [90].

2.6. Red Cell Membrane Damage

The function and viability of red cells is essentially dependent on the ability of the red cell membrane, to maintain its normal cell shape, deformability and mechanical stability [91]. Storage-related alterations in metabolism and oxidative stress have considerable harmful effects on red cell membrane integrity [1]. The red cell membrane consists of a lipid bilayer that is interposed with proteins. The lipid bilayer includes phospholipids, cholesterol and fatty acids that are asymmetrically distributed between the inner and outer layers [70]. Phosphatidylserine is an important component of the lipid layer that under normal circumstances is present entirely on the inner layer, but in senescent red cells, it is expressed on the outer layer of the membrane [70]. When phosphatidylserine is expressed on the outer layer, it is highly thrombogenic and also acts as an essential senescence signal that leads to the removal of red cells by reticuloendothelial macrophages [92]. Cholesterol is another important component of the lipid layer of red cell membrane. Increased cholesterol to phospholipid ratios impair red cell viscosity and deformability and help alterations in red cell shape [70].

Important membrane proteins include; the transmembrane protein anion exchanger 1 (AE1, also known as band 3), and the principal structural cytoskeletal proteins spectrin and ankyrin. The AE1 is a transport protein that regulates the exchange of chloride and bicarbonate across the membrane and also links the lipid bilayer to the cytoskeleton by binding to ankyrin, which in turn binds to spectrin [70]. AE1 plays a role in senescence signaling, as its breakdown can potentially generate a neo-antigen that results in the rapid clearance of the red cell from the circulation [92].

Metabolic changes due to cold-storage and reduced glycolysis as well as increased oxidative stress have profoundly harmful effects on the red cell membrane [1]. Increased auto-oxidation of haemoglobin within the red cell leads to precipitation of structurally distorted forms of methaemoglobin near the cell membrane and causes disruption of AE1 and cytoskeletal membrane proteins [93]. Denaturation of methaemoglobin results in the formation of haemin and iron, which freely divides into the membrane lipid bilayer to cause peroxidation of membrane lipids, proteins, and carbohydrates [94]. Membrane disruption and peroxidation causes membrane cation leakage, increased cholesterol to plasma ratios, phosphatidylserine exposure to outer lipid bilayer, clustering of AE1, and increases production of microvesicles [10]. These changes reduce red cell functionality and viability by causing membrane instability and loss, reduced deformability, alterations in red cell discoid shape and increased senescence signaling [95]. Microvesicles formation and buildup in the supernatant increases exponentially during storage [96]. The accumulated microvesicles, which are haemoglobin-laden and externalize phosphatidylserine, can also cause post-transfusion physiological distresses by contributing to the oversaturation of the body's clearance systems for haemolysed red cells by increasing thrombogenicity due to externalization of phosphatidylserine, and by scavenging of endothelial-derived nitric oxide (NO) gas-which is a strong vasodilator, antioxidant and inhibitor of platelet aggregation -by its component haemoglobin [84]. When haemoglobin comes in contact with Nitric Oxide, it near rapidly consumes the NO to form methaemoglobin and nitrate [84]. Under normal circumstances, encapsulation of haemoglobin within the red cell prevents this reaction because NO does not diffuse well across the red cell membrane [84]. It does readily permeate across the microvesicular membrane, such that the rate of reaction between haemoglobin and NO is increased by 1000-fold [96]. Thus, the accumulated microvesicles in the supernatant can reduce endothelial-derived NO bioavailability in the recipient, which can lead to tissue ischaemia and end-organ injury [96].

2.7. Reduced Red Cell Viability

The final common pathway of injury due to the cold storage-related changes in red cell metabolism, increased oxidative stress and membrane damage may reduce red cell viability resulting in pre- and post-transfusion haemolysis [97]. Pre-transfusion haemolysis leads to the release of red cell cytoplasmic contents in the supernatant, which leads to an approximately eightfold increase in the free haemoglobin levels in the supernatant plasma after 6 weeks of storage [98]. As a result, some of the free haemoglobin in the

supernatant undergoes spontaneous oxidation, resulting in the accumulation of methaemoglobin, haemin, iron and reactive oxygen species in the supernatant, thus provoking the oxidative stress [99]. Methaemoglobin levels, normally constitute <1% of total haemoglobin in the supernatant [70]. However due to storage-related effect, the methaemoglobin level is increased by twofold after 3 weeks of storage [100]. Free iron, which is essentially undetectable during the first few days of storage, is ubiquitous in the supernatant after 3 weeks of storage [101]. Similar to the post-transfusion effects of microvesicles the contents of the supernatant may also reduce endothelial-derived NO bioavailability and contribute to the oversaturation of the clearance systems for haemolysed red cells in the recipient. However, given that the amount of free haemoglobin in the supernatant constitutes <1% of the total haemoglobin in a unit of blood [77], this contribution is likely to be minor [1].

A much more onerous burden on the recipient is likely to be the progressively increasing number of red cells that become senescent but do not necessarily undergo haemolysis during cold storage [1]. These comprise nearly 25% of the total cells in a unit of blood by the fourth week of storage [102] and are haemolysed and removed from the recipient's circulation within 1 hour of transfusion by the reticuloendothelial cells [103].

2.8. Transfusion of Fresh Versus Stored Red Blood Cells

RBC transfusions are used to treat hemorrhage and to improve oxygen perfusion of tissues. Transfusion of RBCs should be based on the patient's clinical condition and should improve the clinical outcomes [69]. Indications for RBC transfusion include acute sickle cell crisis, symptomatic anaemia, or acute blood loss of greater than 1,500 mL or 30 percent of blood volume [69]. Patients with symptomatic anaemia should be transfused if they cannot function without treating the anaemia or if the anaemia is significantly affecting the quality of life of the patient [69]. Common symptoms of anaemia can vary from shortness of breath, fatigue, dizziness, weakness, reduced exercise tolerance, muscle cramps and angina or severe congestive heart failure. Until the 1980s, the 10/30 rule (transfusion when a patient's haemoglobin level is less than or equal to 100 g/L and a haematocrit level of less than or equal to 30 percent) was used as the trigger to transfuse, irrespective of the patient clinical presentation [104].

In 1999, a randomized, multicenter, controlled clinical trial investigated a restrictive transfusion trigger (haemoglobin level of 70 to 90 g/L versus a generous transfusion trigger (haemoglobin level of 100 to 120 g/L in patients who were severely ill [105]. Restrictive transfusion practices resulted in a 54 percent relative decrease in the number of units transfused and a decline in the 30-days mortality rate. The authors recommended transfusion when haemoglobin is less than 70 g/L, and maintenance of a haemoglobin level between 70 to 90 g/L [105]. A recently updated Cochrane review supports the use of restrictive transfusion triggers particularly in patients who do not have cardiac disease [106].

A similar study was carried out in severely ill children [107]. The restrictive transfusion trigger was a haemoglobin level of 70 g/L with a target level of 85 to 95 g/L. The liberal transfusion trigger was a haemoglobin level of 95 g/L with a target level of 110 to 120 g/L. Patients in the restrictive group received 44 percent less blood transfusions, with no difference in rates of multiple organ dysfunction syndrome or death. The restrictive transfusion strategy seems useful for children who are stable patients in intensive care. It is however contraindicated in preterm neonates or in children with severe hypoxemia, active blood loss, haemodynamic instability, or cyanotic heart disease [108].

Red blood cells (RBCs) degrade progressively during the weeks of cold storage. There is no universally accepted definition of 'fresh' or 'old' blood [108]. While practices vary from country to country, the use of preservative solutions allowing for a shelf life as long as 7 weeks have been licensed. Transfusion of stored RBCs especially those at the end of the approved shelf life have been associated with adverse clinical outcomes. The results of observational analyses in animal models and studies in volunteers have proved provoking, divisive and conflicting [108]. A recently completed randomized controlled trial (RCT) in premature infants illustrates the difficulties with moderately sized clinical studies. Several other RCTs are in progress. The effect of RBC storage seems to vary according to the clinical setting. Resolution of the importance of red cell storage lesion may require large realistic clinical trials [109].

About 90 million units of red blood cells (RBCs) are transfused worldwide annually. Transfusion of RBCs saves lives and enables many medical therapies and surgeries to be carried out successfully. RBCs meet the definition of an essential medicine, although its use became widespread before and without the rigorous evaluation of randomized clinical trials [69] [69]. Despite well exposed, immunomodulatory, immunological and rare infectious adverse events [110] in developed countries, RBC transfusion seems to have a therapeutic index exceeding that of many common medications [69]. However, RBC shelf life is determined and stringently regulated not on the basis of clinical studies but based on in vitro haemolysis and in vivo radiochromium label recovery and survival studies [48]. The acceptable limits were originally derived from expert opinion and not necessarily through correlation with clinical outcome [5].

Red blood cell transfusion for surgical and severely ill patients remains vital, and for some life-threatening situations, the only medical approach for treating clinical symptoms related to haemorrhage and anaemia [111]. The impact of transfusing fresher vs older red blood cells (RBCs) on patient remains controversial [111]. For almost 20 years, retrospective and prospective observational studies have shed light on the harmful effect of RBC storage [112]. A large retrospective single-centre study comprising 6002 patients ([113] raised both interest and concern about the quality of

stored RBCs and their potential toxicity. A formal meta-analysis of observational studies and RCTs focusing on patient survival rates established that irrespective of clinical situation, trial size or volume of blood transfused, older stored blood was linked with a significant increase in mortality [114].

Studies in controlled animal models have demonstrated that longer storage of RBCs leads to morphologic changes that could have a harmful effect on microvascular perfusion and by implication tissue perfusion. These changes include changes to the cell shape, membrane, an increase in adhesiveness, a decline in flexibility and reductions in capillary flow [115]. Further, older blood is associated with release of free iron that may predispose to vascular dysfunction, thrombosis, and nosocomial infections [116].

Storage medium could have a harmful effect through the generation of superoxides and inflammatory mediators that could result in oxidative stress and damage [117]. Observational studies, although potentially confounded have suggested that these complicated mechanisms (referred to as red cell storage lesion) may negatively affect patient-important outcomes including infection, organ failure, hospital stay, and death [110].

Prior evidence on the age of transfused RBCs has been dominated by uncontrolled observational studies that suggest better results with fresher RBCs [118]. However, a recently published 2015 Cochrane Systematic Review that focused exclusively on 16 randomized controlled trials was unambiguous in their findings. This was a methodologically strong review, but because of lack of uniform definitions of fresher or older RBC storage and overlap in the distribution of the age of RBCs, the authors did not carry out any meta-analyses and noted that limitations in the evidence precluded definitive conclusions [109]. In 12 trials (involving 5229 subjects) in which 6 compared fresher RBCs with older RBCs and 6 compared fresher RBCs with current standard practice. There was little or no impact of fresher versus older RBCs on mortality or on adverse events [111].

There is a scarcity of reliable prospective controlled clinical data regarding the safety and efficacy of RBC transfusion and there are amazing gaps, despite the fact that 142 related RCTs published up to 2009 [119]. Eleven RCTs evaluated RBC storage times with a median of 35 patients and only two RCTs investigated clinical outcomes including post-operative bleeding and mortality in a trial published subsequently [120]. Physiological factors, rather than mortality were the primary outcomes in most of the completed RCTs. None of these RCTs detected any effect of RBC storage time on clinical results [109]. Hence, any guideline limiting the age of RBC units at transfusion is largely based on expert opinion and often organizational policy reflecting their understanding of the basic principles of RBC function and survival [56].

Guidelines in some healthcare systems are more rigorous than the accepted standard of care and require transfusing fresher RBC units to vulnerable patients including those requiring intrauterine transfusion, premature infants or newborns, patients with sickle cell disease or cardiac surgery interventions or any patient with chronic transfusion need [56]. These patients may receive the freshest RBC unit in inventory but not older than 7 days [121] or 10 days based on the hypothesis that these RBCs function better than their older counterparts, improving clinical outcome. Although these guidelines may have long traditions seem to be instinctive and could prove to be beneficial for patients. Evidence is however lacking or inconclusive that clinical outcome is affected by age of RBC units [56]. In the US, there is no guidance in the Standards for Blood Banks and Transfusion Services, 27th Edition 2011, or endorsement in the Technical Manual for maximum length of storage before transfusion in any adult patient group [122].

Juffermans and colleagues [123] reported in a small retrospective cohort study that involved 196 patients admitted to an adult intensive care unit (ICU) over a period of almost 4 years. RBC units were categorized as being ≤14 days versus >14 days old. Infection was used as the endpoint in this study and an original feature for any study comparing fresh versus old RBC units. Previously, either mortality or multiple organ dysfunction score (MODS) was used as the endpoint in most of the existing RCT studies [124]. Mortality was also an endpoint in two other retrospective studies, which seem to refute each other. One that patient survival was [124] or was not [125] associated with the use of stored RBC in these retrospective studies comprising 6,002 and 670 subjects. However, these two reports in 2008 ignited a significant interest in blood storage lesion and its potential negative effect on clinical outcome [125].

The first of the larger RCTs that investigated mortality as the primary outcome was recently completed [120]. The use of fresh RBCs compared with standard blood bank practice did not seem to improve major neonatal morbidities (bronchopulmonary dysplasia, necrotizing enterocolitis, retinopathy of prematurity, intraventricular haemorrhage or death in premature subjects with very low birth weight infants requiring a transfusion. However, the average duration of RBC storage in the standard of care group (14. 6 days) does not reflect the average RBC storage in the US (18 days) and definitely not the practice of some centers that routinely store RBCs for 21 days or longer [126]. The five-large ongoing RCTs use mortality or multiple organ dysfunctions as primary outcomes and study fresh RBC versus standard of care. The patient cohorts studied will be important for the implementation of the results and include all acute care inpatients, severely ill patients in adult intensive care units and patients with complex cardiac surgery. The results of these large RCTs are expected in the next few years [109]. Current evidence provides modest conviction that the use of fresher RBCs does not influence mortality, and low certainty that it does not influence adverse events but may increase infection rates. The current evidence does not seem to provide any support for changing practices toward fresher RBC transfusion [111].

2.9. Pre-clinical Studies with Volunteers

The effect of fresh-versus-old RBCs on physiological parameters can be evaluated reliably and safely by transfusing autologous RBCs to healthy volunteers. Such studies unlike clinical trials permit maximal separation of the age of stored RBCs. No differences were found for cognitive and pulmonary function or hyperaemia in three different volunteer studies. The informative study by [127] documented highly significant increases of serum iron and transferrin saturation at 4 hours (h) after transfusion of older RBCs. Ferritin concentrations increased from baseline only after transfusion of older RBCs. While non-transferrin-bound iron concentration was not significantly increased after fresh RBCs. However, it progressively increased until 4 hours after transfusion of older RBCs. The ongoing haemolysis during cold storage of RBC may explain the significantly increased serum total bilirubin peaking at 4 hours, which correlated with a peak of unconjugated bilirubin for some volunteers and a small, but significant rise in serum conjugated bilirubin. While the results from volunteers raise few issues, the volumes transfused are small and the recipients healthy so the effects may be quite different in critically ill patients. The exact study details, such as RBC preparation and volumes transfused, need to be considered when extrapolating from these convincing data [109].

2.9.1. Inadvertent Consequences of Using Fresher RBCS

A transfusion policy with fresher RBCs could limit the blood supply in critical situations for some patients. Adverse effects of transfusion are strictly monitored, but adverse effects caused by an inadequate blood supply are significantly difficult to measure at the cumulative level [56]. Patients in critical need can suffer a negative clinical outcome by either the lack of fresher RBCs or by availability of blood that is not ideally matched by blood group. Such a circumstance would reverse any benefit gained from transfusing a slightly fresher RBCs for the majority of patients [56].

Transfusing fresher RBCs might benefit some patient cohorts, such as severely ill patients in intensive care units or patients with infections, while offering a limited or no benefit for the majority of other patients. If fresher RBCs were generally transfused without restricting the RBC shelf life, older RBCs might be reserved for certain patients, such as those with trauma and extensive surgical procedures requiring large amounts of RBCs. However, these patients might benefit most from fresher RBCs. Some neonatologists avoid transfusing the freshest RBCs by using aliquots drawn from a single donor when limiting donor and infectious disease exposure is considered the superior objective. Some school of thought believe that the transfusion of fresh RBCs and limiting donor exposure may not be conflicting goals if the shelf life is 28 days or shorter [128].

A review of the reports on transfusion-dependent patients concluded that several studies indicate that fresh RBC might be associated with adverse outcomes [129]. In a retrospective study, the same group reported an almost two-fold increase in mortality rate after transfusion of fresh compared to older RBC [130]. The proposal that RBCs with a shelf life of 5–21 days may have any negative effect, worse than older RBCs, is not instinctive and has not been widely shared [109].

Implementing and maintaining any policy of fresher RBCs may require major operational changes to the current practice of collection, storage and transfusion in developing low income countries. Recruiting additional blood donors, increasing RBC outdating or more complex inventory management will increase costs. For changes that involve major practical and organizational consequences, such as reduction of the RBC storage time, a multi-tiered, stepwise and carefully monitored approach seems prudent particularly in developing countries where there is a multi-dimensional challenge associated with safety, adequacy and access to ad equate resources to fund the major operational changes associated with the implementation of fresher RBCs policy [109].

2.9.2. Current State of Clinical Evidence on the Clinical Relevance of the Red Cell Storage Lesion

There has been a significant number of studies investigating the effects of prolonged blood storage on clinical outcomes. These have included healthy volunteers, numerous retrospective and prospective observational studies and a handful of older, more recent and ongoing randomized controlled trials [127]. Overall, study results have been conflicting and affected by multiple procedural issues including confusing, methodical bias, limited external validity and multiple statistical deficiencies [1].

There are a number of clinical data primarily from retrospective studies on red cell storage lesion [131]. Additional retrospective studies in different clinical situations appear on a regular basis [132]. However, despite the numerous studies, blood transfusion experts are left with divergent results for clinically important outcomes [109].

Among the 32 studies tabulated by one review [131], a significant number (18) reported a harmful effect of prolonged RBC storage, while 14 did not. The seven-prospective observational or case–controlled studies were evenly distributed (four with and three without significant harmful effect). However, the four prospective randomized studies in this tabulation showed no significant harmful effect [124].

One report tabularized 55 studies including eight small RCTs [113]. The 47 non-RCT studies investigated clinical outcomes including mortality (22 studies); occurrence of infection (18); organ failure (12); tissue oxygenation and microcirculation (11); length of hospital stay (9) and other outcomes (8). The significant heterogenicity and procedural flaws of the included studies prevented the authors from combining the data or trying to determine the reasons for the different observed effects [133].

The largest retrospective cohort comprised 364,037 patients, and concluded that the excess mortality if any,

caused by older RBC is probably <5% [133]. Among the four next largest studies, two studies comprising 6002 [114] and 4,933 patients [134] reported a harmful clinical outcome, while the two other studies, comprising 3475 and 2732 patients [129] did not. Other possible factors such as variations in leucocyte depleted RBC blood products or ABO matching are of concern and not always controlled or even documented in detail [109].

The retrospective and small prospective studies raise concerns about the efficacy and toxicity of older stored RBCs. However as important as they are in postulating hypothesis, the data are often not sufficiently robust to warrant changes in national blood policies. In retrospective studies, transfusions may represent no more than a marker of illness severity independent of other parameters, like the APACHE II (Acute Physiology and Chronic Health Evaluation II) score. The known confounding factors are difficult to adjust no matter how much effort authors put into statistical analysis. Furthermore, publication prejudice favoring positive studies is an unfortunate fact. Large pragmatic RCTs are the current 'gold standard' in evidence-based medicine that may lend a better insight into effects of prolonged blood storage on clinical outcomes [109].

3. Conclusion

Although blood collection and administration are safer and more efficient than ever before, red cells have been shown to undergo multiple metabolic and structural changes during storage that may compromise their functionality, viability and associated adverse effect following transfusion. The clinical relevance of these changes is a hotly debated topic that continues to be a matter of intense investigation. Current evidence provides moderate certainty that use of fresher RBCs does not influence mortality, and low certainty that it does not influence adverse events but could possibly increase infection rates. The existing evidence provides no support for changing practices toward fresher RBC transfusion. This seems an advantage particularly in developing countries where a major challenge of inadequate supply exists to meet the increasing need of red cells. Storing blood longer can potentially minimize the wastage of scare allogenic blood due to expiry.

Recommendations

There is need for the establishment of criteria for red cell storage that include or consider the clinical outcomes of receiving fresh or old red blood cell.

We recommend that the blood banking system should establish a means of checking or screening for the degree of haemolysis of stored red cell prior transfusion.

We advocate on the use of additive solution that store red cell for longer period (for example SAG or SAGM) than what it is being used (CPDA-I) particularly in developing countries.

There is the urgent need for the development of more optimum storage solution that can potentially allow for the effective and long-term cold storage of RBC with less associated haemolysis.

There may be need to provide fresher red cells for some patients' groups particularly neonates and patients in critical areas like ICU and HDU.

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