




# The Future of Red Cell Transfusion Lies in Cultured Red Cells

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Ind J Med Paediatr Oncol 2021;42:528–532.

## Abstract

Blood is a very important resource for healthcare-based services and there has been a consistently increasing demand for it in most parts of the world. Poor volunteer-based collection system, high-risk of transfusion-transmitted infections, and emergence of new pathogens as evident from the ongoing Coronavirus Disease 2019 (COVID-19) pandemic are potential challenges to the global healthcare systems. It is imperative to explore safe and reliable alternatives to red cell transfusions. Ex vivo culture of red cells (cRBCs) from different sources such as hematopoietic stem cells (HSCs), pluripotent stem cells, and immortalized progenitors (e.g., BELA-2 cells) could revolutionize transfusion medicine. cRBC could be of great diagnostic and therapeutic utility. It may provide a backup in times of acute shortages in patients with rare blood groups, and in cases with multiple antibodies or sickle cell anemia. The CRISPR-Cas9 system has been used to develop personalized, multi-compatible RBCs for diagnostic reagents and patients with multiple allo-antibodies. cRBC could be practically feasible for pediatric patients, who require small quantities of red cell transfusions. cRBC produced under good manufacturing practice (GMP) conditions has been reported to survive in human blood circulation for more than 26 days. Recently, a phase I randomized controlled clinical trial called RESTORE was initiated to assess the survival and recovery of cRBCs. However, feasible technological advancement is required to produce enough cRBCs for clinical use. It is crucial to identify sustainable sources for large-scale production of clinically useful cRBCs. Although the potential cost of one unit of cRBC is extrapolated to be around US\$ 8000, it is a life-saving product for patients having rare blood groups and is a “ready to use” source of phenotype-matched, homogenous young red cells in emergency situations.

## Keywords

- ▶ cultured red cells
- ▶ BELA-A2 cells
- ▶ clinical significance

DOI <https://doi.org/10.1055/s-0041-1740068>.  
ISSN 0971-5851.

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## Introduction

Blood is a very important resource for healthcare-based services. Although the demand profile varies in different parts of the world, there has been a consistently increasing demand for blood. In the developed world, the increase in the demand is attributed to longer life expectancy and increase in complex medical procedures, arising from cardiovascular surgeries and oncology treatments, whereas the common causes of red cell transfusion in the developing world are post-partum hemorrhage and severe childhood anemia. The high risk of transfusion-transmitted infections and emergence of new pathogens (such as severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) has compounded the problem of safe blood supply shortages.<sup>1</sup> The World Health Organization (WHO) Factsheet 2013 reveals that despite 112.5 million blood donations globally every year, there are around 100,000 deaths annually in the developing countries due to unmet transfusion needs of pregnant women alone.<sup>2</sup> Poor volunteer-based collection system in developing nations and problems with transient supply shortages in the developed world are potential challenges to the global healthcare systems. It is imperative to explore safe and reliable alternatives to red cell transfusions.<sup>3</sup>

We aimed to review the current literature on cultured red cells and its possible clinical applications.

## Methodology

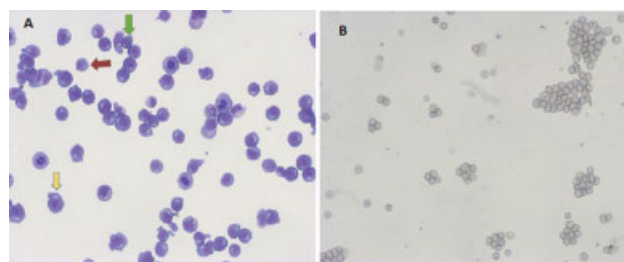
PubMed and Google databases were searched for effective methods of red cell cultures and its possible future clinical use. The keywords used for literature search included red cell cultures, BELA-A2 cells and transfusion. Reference lists were cross-checked for relevant citations, and more searches were undertaken until the desired information was obtained.

## Cell Cultures

*Ex-vivo* red cell cells (cRBCs) could revolutionize transfusion medicine. cRBCs could be derived from different sources such as hematopoietic stem cells (HSCs), immortalized progenitors and pluripotent stem cells. cRBCs could provide a crucial backup during acute shortages in rare blood groups and in cases with multiple antibodies. It is imperative to identify reliable, safe, and sustainable sources for large-scale production of clinically useful cRBCs.

## Normal Hematopoiesis

Normally, the HSCs differentiate into a common myeloid lineage, megakaryocyte-erythroid precursors, and subsequently to erythroid precursor cells such as burst-forming units (BFU-E) and colony-forming units (CFU-E).<sup>4</sup> The use of erythropoietin is essential for the multiplication of erythroid progenitors making them sensitive to the effect of other growth factors during hematopoiesis. It takes 7 days for the CFU-E to proliferate and differentiate into mature erythroblasts.<sup>5</sup> In the presence of cytokines, erythropoiesis progresses with the development of proerythroblasts,



**Fig. 1** (A) Microphotograph of erythroid cultures showing proerythroblasts (yellow arrow), basophilic erythroblasts (green arrow), and polychromatic erythroblasts (condensed chromatin with gray-blue cytoplasm, red arrow) on Day 7 (20x, Giemsa stain). (B) Microphotograph showing reticulocytes in clusters and discrete after filtration of erythroid cultures (20x, Giemsa).

followed by basophilic to polychromatic erythroblasts, and finally to orthochromatic erythroblasts that enucleate into reticulocytes (→ Fig. 1).<sup>6</sup> Toward the terminal differentiation, the expelled nucleus of the erythroblasts is phagocytosed by macrophages. Presently, laboratory scientists are exploring multiple ways to replicate hematopoiesis *in vitro*.

## Liquid Culture Methods

Hematopoiesis was initially studied on a semisolid culture medium, but it was difficult to quantitatively analyze the growth kinetics, and biochemical and immunological characteristics of immobilized developing cells. Fibach et al developed the two-step liquid cultures to study erythroid cell development.<sup>7</sup> In the first step, erythroid progenitors from the umbilical cord and adult peripheral blood were amplified using medullary microenvironment, comprising stromal cells, and subsequently, these cells underwent maturation.<sup>6</sup> Douay et al did several studies under well-defined conditions to amplify stem and progenitor cells from cord blood.<sup>8-10</sup> Thereafter, several researchers developed a modified cocktail of cytokines called SED, which comprised stem cell factor (SCF), erythropoietin (EPO), and dexamethasone. This cocktail helped induce the growth of progenitors cells and their differentiation into stress erythroblasts. The appearance of stress erythroblasts is usually seen during major blood loss and are considered the first line of defense.<sup>11-13</sup> Migliaccio et al modified the SED cocktail to cause massive amplification of cRBCs that had antigens similar to human RBCs *in vivo*.<sup>14-16</sup> Lately, self-renewing erythroblasts were isolated from early mouse embryos. These primary erythroid precursors extensively proliferated *ex vivo* on exposure to SED cocktail ( $10^6$  to  $10^{60}$ -fold) and have renewed interest among scientists to search for similar cells in humans. It is believed that these self-renewing erythroblasts could serve as a potential semi-permanent source of cRBCs in the future.<sup>17</sup> Based on the number of media stages (2 to 4), culture systems could be classified and their difference lies in the addition and subtraction of several cytokines (→ Table 1). In general, the primary stage causes the expansion of HSCs in the presence of EPO, SCF, and interleukin-3 (IL-3). In the secondary stage, cytokines such as SCF, EPO, and transferrin cause secondary expansion of erythroblasts. EPO and

**Table 1** Types and contents of culture media for erythroid cells

<b>Primary Medium</b>
Iscove's modified Dulbecco's media (IMDM) with glutamine, SCF, IL-3, EPO, AB serum, insulin, heparin, holotransferrin, and
HAS
<b>Secondary Medium</b>
As primary media excluding IL-3
<b>Tertiary Medium</b>
As primary media excluding IL-3 and SCF

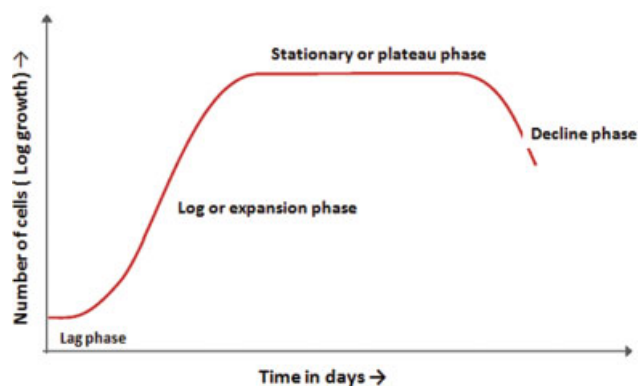
Abbreviations: AB; EPO, erythropoietin; HAS; IL, interleukin; SCF, stem cell factor.

holotransferrin are responsible for the terminal differentiation. Several other cytokines such as IL-6, thrombopoietin (TPO), and Feline McDonough Sarcoma (fms)-like tyrosine kinase 3 (Flt-3), along with glucocorticoids<sup>18</sup> could be added to the first stage to facilitate the expansion before the differentiation. Researchers have also found that early erythroid progenitors self-renew in the presence of glucocorticoids, along with many agonists such as prolyl hydroxylase inhibitors,<sup>19</sup> transforming growth factor  $\beta$  (TGF- $\beta$ ), and peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ).<sup>20,21</sup>

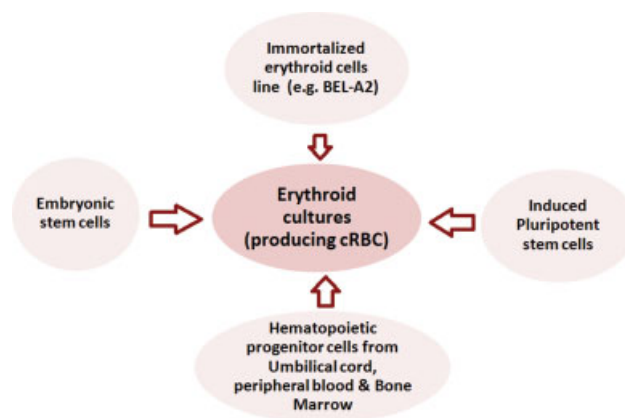
## Growth Curves of cRBC

Normally, cRBCs show a typical sigmoid pattern of proliferation. The hematopoietic progenitors undergo a lag phase, in which the cells do not divide but adapt to the culture conditions.<sup>22</sup> Lag phase is followed by logarithmic (log) growth phase, characterized by active proliferation and exponential increase in cell density and eventually the cells enter the stationary or plateau phase, which reflects the slowing down of cell proliferation as they become confluent ( $\rightarrow$  Fig. 2). The initial progenitors could be derived from the sources ( $\rightarrow$  Fig. 3) discussed below.

Circulating HSCs and progenitor cells derived from healthy human donors and cord blood collections have successfully been cultured. This could be very useful to



**Fig. 2** The growth curves showing the cumulative increase of erythroid cell cultures (number of cells in the y-axis) over the a period of time (days) on the x-axis.



**Fig. 3** Methods of generating cRBCs. cRBC, culture of red blood cells.

expand the blood supply of rare blood groups. Theoretically, HSCs in a single unit of cord blood could be amplified to make 500 units of RBCs. Because circulating HSCs and progenitor cells have limited proliferative potential, the production of cRBCs depends on a donation-based collection system. The testing of these cells for transfusion-transmitted infections increases the cost and does not completely eliminate the risk of emerging pathogens.<sup>6</sup> cRBCs also have a short half-life; it is required to further optimize the culture conditions. Another drawback of these primary cultures is their genetic variability that influences their growth patterns.

Immortal cells such as pluripotent stem cells (human embryonic stem cells [hESCs]) and induced pluripotent stem cells (iPSCs) can also differentiate into erythroid lineage using human feeder layers. Feeder cells derived from a human or animal source do not divide themselves but allow unlimited proliferation of other cells by secreting growth factors. Genetic engineering and xenobiotic-free method could expand the use of these cells in future.<sup>6</sup>

## Immortalized Erythroid Cell Line BEL-A2

Early adult erythroblasts are immortalized to produce a stable cell line, which generates an unlimited supply of cRBCs. Immortalized progenitors are currently the focus of research in transfusion medicine as they could be cultured in relatively simple media. On induction, these cells have the potential to differentiate into cRBCs.<sup>6</sup> Trakarsanga first reported the human immortalized cell line called Bristol erythroid line adult (BEL-A2).<sup>3</sup> The advantage of BEL-A2 is that it avoids long differentiation time (unlike pluripotent stem cells) and does not require constant donations (unlike umbilical cord blood or peripheral blood stem cells). BEL-A2 cells were created using the Tet-inducible human papilloma virus-16-E6/E7 expression system. Their origin lies in CD34<sup>+</sup> cells of the adult human bone marrow that were cultured for 4 days in a primary medium for erythroid cells. E6 and E7 protein expression was induced using doxycycline on the fifth day. In continuous culture, the doubling time of BEL-A2 cells was 20 hours after Day 100. The cells underwent normal erythropoiesis and showed no functional or molecular difference to ex vivo cultured reticulocytes ( $\rightarrow$  Fig. 1). However,

enucleation of BEL-A2 cells into mature reticulocytes is around 30%, which is far from optimum.<sup>3</sup>

Whole exome sequencing (WES) has been done to analyze BEL-A2 genotype, particularly genes associated with blood groups and enucleation. Based on the WES data, blood group phenotypes were correctly predicted and showed concordance with serological results.<sup>3,23</sup> The WES data of BEL-A2 could be used for manipulating blood group-related genes by gene editing strategies (such as CRISPR-Cas9). In future, antigen-edited red cells could be of diagnostic utility in transfusion laboratories, and rare blood group cells could be expanded for therapeutic purposes.<sup>23</sup>

## Large-Scale Production of cRBCs

In 2011, the transfusion of cRBCs was proven in principle by Giarratana et al, who produced these cells under good manufacturing practice (GMP) conditions and labeled them with <sup>15</sup>Cr.<sup>8</sup> It was observed that 40 to 60% of the cRBCs remained in the blood circulation of healthy human volunteers on Day 26 after injection comparable to the half-life (28 days) of native RBCs. It has been reported that purified cRBCs could be stored for 4 weeks at 4°C in Sg-M (saline adenine glucose mannitol)-based solutions.<sup>8</sup> Recently, the University of Bristol and the University of Cambridge have initiated a clinical trial called RESTORE involving cRBC. It is a phase I, randomized controlled crossover trial among healthy human subjects to assess the survival and recovery of cRBC.<sup>24</sup> Although there has been some progress in cRBC production, it would require feasible technological advancement to produce enough cRBCs for clinical use. Presently, more than 1000 L of medium is required to produce 1 to 2 × 10<sup>12</sup> cells, contained in a unit of blood.<sup>6</sup> Bioreactors are being employed for scaling up cRBC production.<sup>25</sup> Bioreactors mimic hematopoietic microenvironment with biomimetic materials having a continuous perfusion conditions as in the bone marrow. In a bioreactor, an artificial niche comprises a scaffold macroporous structure (polymer matrix), and mesenchymal stem cells are created and subsequently, HSC seeding is done. The conventional two-dimensional (2D) culture system is being replaced by a 3D reactor, which could maintain HSCs for a longer period of time, and hence, optimize large-scale cRBC production to 10 L in 3 to 4 weeks per reactor.<sup>26</sup>

## Clinical Significance of cRBCs

In the near future, cRBCs could be used to manufacture reagent RBC cell panels of known antigen profile used for testing patient serum having unknown antibodies. Present cell panels mostly cover antigen profile of Caucasians; hence these lack greater diversity of antigens to detect uncommon African and Asian phenotypes. Thus, the availability of these reagent RBC panels could greatly improve transfusion services for multi-transfused patients, likely to have multiple alloantibodies. Multiple blood transfusions often lead to alloimmunization caused by incompatibilities at several minor blood group antigens. Hawksworth et al has demonstrated the use of gene-editing strategies on immortalized

**Table 2** Advantages of cRBCs

• cRBC are homogeneously young and have a longer life span than native RBCs (heterogeneous in age)
• Improved transfusion efficacy as the product has homogeneously young cells
• Personalized antigen-negative red cell transfusions for patients with multiple allo-antibodies
• Provision to produce “rare” blood groups
• Reduces the risk of allo-immunization
• Greatly reduces the risk of transfusion-transmitted infections
• Inventory management of red cell units is reliable and donor independent
• Diagnostic use in transfusion laboratories

erythroid cell line using the CRISPR-Cas9 system to knockout several antigen coding genes and generate null phenotype red cells. Hence, these personalized, multi-compatible RBCs could be potentially used for diagnostic reagents and patients with multiple allo-antibodies.<sup>27</sup>

Rare RBC phenotypes lack high-frequency antigens on their surfaces and sometimes, characterized by the presence of antibodies against these antigens (e.g., Bombay blood group or p phenotype of P blood group). Hence, they require only identical rare RBCs for transfusions. The culturing of rare red cell phenotypes could be beneficial to such patients, especially pediatric patients, who require small quantities of red cell transfusions.<sup>28</sup> The application of cRBCs could be extended to antigen-negative red cell units for patients with multiple antibodies or sickle cell anemia. In hemoglobinopathies, it is preferred to transfuse RBCs of a prolonged life span to reduce the frequency of transfusions. Improvements in culture techniques help generate cRBCs having a long life span that could be used in patients (► **Table 2**).

Because storage lesions of RBCs adversely affect the morbidity and mortality in intensive care patients, it is postulated that RBCs generated under completely standardized conditions are metabolically stable and would considerably reduce storage lesions.<sup>29</sup> It is believed that cRBCs would be deficient in leukocytes and related cytokines; hence it would greatly reduce the risk of transfusion reactions such as non-hemolytic febrile reactions and TRALI (transfusion-related acute lung injury).

## Cost of cRBC Units

Several studies have extrapolated the cost of producing 1 unit of cRBC to be around US\$ 8000. Although the price of cRBCs is exorbitant, there is an ongoing debate in the developed world to invest in a life-saving product for patients having rare blood groups or those with multiple allo-antibodies. Ready to use cRBC units, having a homogeneous population of young red cells, could obliterate the need for frequent transfusions and frantic searches for phenotype-matched blood in emergency situations.<sup>30,31</sup>

## Conclusion

cRBCs have the potential of revolutionizing transfusion medicine, as these generate a reliable supply of homogenous RBC concentrates having a selected phenotype, with a long life-span and free of leukocyte contamination. If clinical trials have a favorable outcome, the routine use of cRBCs is expected to overcome engineering and economical hurdles. The reality of cRBCs would materialize in the clinics, if technological advancements takes place in a time-bound and cost-effective manner.

### Authors' Contributions

Rizwan Javed and Deepak K. Mishra contributed to conceptualization of the study. Lorraine Flores and Saurabh Jayant Bhavne contributed to the design of the study. Saurabh Jayant Bhavne and Asheer Javed defined the intellectual content. Rizwan Javed, Asheer Javed, and Lorraine Flores contributed to literature research. Rizwan Javed contributed to manuscript preparation. Lorraine Flores, Asheer Javed, and Deepak K. Mishra contributed to manuscript editing. Saurabh Jayant Bhavne, Asheer Javed, and Deepak K. Mishra contributed to manuscript review.

### Funding

We would like to thank the Commonwealth Scholarship Commission in UK, London, and University of Bristol for funding the training of the first author in Transfusion and Transplantation Sciences (including studies on red cell cultures) at NHS Blood and Transplant at Filton.

### Conflict of Interest

None declared.

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