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The secrets of human stem cell-derived transfusable RBC for targeted large-scale production and clinical applications: A fresh look into what we need most and lessons to be learned

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ABSTRACT

Blood transfusion, using the safest conventional blood bioproducts, is an irreplaceable part of substitution therapy. It is considered the most essential supportive clinical intervention aimed to restore the health of patients in need. Nevertheless, numerous unresolved problems are still associated with current blood substitution therapy. To alleviate our dependency on blood donors, many investigators have been focusing on the quest for stem cell-derived blood cells in line with major developments in the field of regenerative medicine. The main objective is to provide a safe and highly standardized universal cultured red cell concentrate [CRBC] for all clinical applications, regardless of blood groups. Currently, we are close to overcoming some of the main obstacles in culturing cells. This concise report is a prelude to the immortalized cell lines that are ready for *in vivo* clinical trials. It is only through the sharing of experimental ideas and knowledge-based strategies that we will be able to achieve such an enormous task and better understand ''the one for all concept'' of CRBCs and their universal usage in all clinical settings.

1. Background

Numerous unresolved problems hamper conventional blood substitution programs including: a] the paucity of donors and their associated physiological, immunological and hematological variabilities, b] the ever-dynamic nature of transmission of pathogenic microorganisms due to the “window period” for known and emerging pathogens, as exemplified with the unexpected arrival of the pandemic outbreak of coronavirus disease [COVID- 19], leading to shortage of a safe and adequate blood supply, c] the need for multilayers of preventative measures using the upgraded pathogen reduction technologies for each unit of blood, with concerns regarding operational time and economic impact, and finally, d] the trend for increasing life expectancy of our aged populations, that are predicted to require more transfusion products than currently supported by voluntary donation along with the ever increasing need in both military and civil trauma cases.

To overcome these concerns some investigators have been looking for alternative blood donor-independent supply strategies, in line with the concept, that in a safe society, the patients' safety must be paramount and secured. Hence, the focus has moved to the industrial *ex vivo* production of highly standardized and universally transfusable cultured red blood cell [CRBC] products capable of substituting conventional packed red blood cells. This concept is now approaching fruition, through R&D studies in many institutions in parallel, that follow the *in vivo* physiological mechanisms of erythropoiesis, and advances in the fields of collection, culture, preservation and expansion of hematopoietic stem cells

The currently used sources of progenitor cells are all indicating that, with some refinement in the study protocols, adequate numbers of CRBCs can be obtained *in vitro* and that the observed economic and biotechnological obstacles of the recent past can be overcome [1,2]. Clearly, unlocking the secrets of the highly standardized CRBC production requires more targeted innovative R&D studies. However, progress among several international institutes, including those associated with the UK NHS BTS [Filton in Bristol and Cambridge University], has been achieved. Whilst the Scottish NHSBT group has abandoned their most exciting innovative projects on CRBC due to instability of their expansion media, there are important lessons to be learned from their research program.

From a practical standpoint, a CRBC project must be based on establishing the same functional capabilities of circulating red cells or at least of RBCs stored for transfusion purposes. Physiologically, RBCs evolved to have high concentrations of haemoglobin and metabolic/redox enzymes in their cytosol, to achieve efficient transportation of oxygen and provide antioxidant defense potential throughout the body. The gradual accumulation of irreversible lesions targets natural RBCs for prompt removal by the reticuloendothelial system. This evolutionary adaption, however, is no longer effective when RBCs are stored hypothermically in citrated anticoagulant or storage media that optimally enhance their functional integrities for longer periods. Most of these donor dependent, storage-induced cumulative damages in red cell shape and function, that develop during the shelf life of stored RBCs, become irreversible after 21 days [3] and such changes might have considerable clinical consequences and significant immunomodulatory impact [1–4]. The potential causes and their clinical consequences as well as the main parameters involved in the storage lesion have been recently identified and supported by newer data obtained from the modern "omics". While the physiological consequences of storage lesions on *in vitro* studies are well established, the potential: a] link to clinical outcome and b] interventions to mitigate the extent of the storage lesion development, are still subjected to randomized controlled trials based on the age of stored blood and clinical outcomes [1,4]. On

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one hand, these studies suggested that the age of stored RBCs is neutral in terms of transfusion efficacy, mortality, morbidity and adverse effects to the recipients [5–7]. On the other, they also highlighted that since the presence of significant variation in the transfusion outcome cannot be attributed to the age of transfused blood it must be linked to – undefined up to date – parameters, probably attributed to donor characteristics and recipient status. To support, when comparing storage lesion parameters in RBC units generated from donors with different genetic background, large differences occur regardless of the storage period examined [8–10]. Clearly, further data and extensive analyses on the quality, safety and efficacy of CRBC are needed in order to determine the suitability and superiority/inferiority of any available CRBC products in comparison to conventional components, especially if these products are to be stored – even for a short period – at blood bank conditions prior to their administration to patients.

2. Cultured RBCs: from R&D phase to clinical trials

The innovative concept of culturing blood cells using human adult peripheral blood $CD34^+$ cells began almost 4 decades ago [11]. Today, many experts in the field have switched their efforts to generation of erythrocytes from stem cells, as the primary source material, using relevant expansion media. Objectively, if successful, such a process would improve the availability of much safer, matched blood cells in terms of adherence to standardized quality criteria, and provide an almost unlimited supply of much needed bioproducts. A universal CRBC product would be ideal for treating patients with various pathophysiological problems, in particular those having rare blood phenotypes, and for reducing alloimmunization and immune-modulation, still unresolved problems in transfusion medicine. In fact, phosphatidylserine (PS) exposure, the end mark of apoptosis in human erythrocytes is dependent on *in vitro* cold storage, while the presence of PS^+ extracellular vesicles has been implicated to transfusion related immune modulation [12].

While cultured RBCs grown in the laboratory have already been used for experimental purposes, the clinical use of such *ex vivo* generated RBCs requires that they are functionally equivalent to native RBCs. This has not been accomplished yet, despite the fact that these methods are moving towards good manufacturing processes before application to the clinical setting.

Current efforts to produce cultured RBCs have generated at least three major questions that need to be addressed: a] identifying the most appropriate source of human stem cells; b] suitability, and in particular, long-term stability of culture conditions and; c] immortalization of progenitor cells for large scale industrial production facility fit for purpose. In this context, we do not know in depth the detailed biological picture of the erythropoiesis process, so, we are still walking blindfolded into a highly complex system.

3. Fundamental manufacturing and quality assessment of CRBC

It is important to highlight that the physiological regulation of the production of erythroid lineage cells from pluripotent stem cells, is a highly regulated system. During differentiation, progenitor cells undergo substantial changes not only in size but also in haemoglobinization and chromatin condensation in order to be ready for enucleation. Nuclei are extruded to form reticulocytes, which enter the circulation and mature into young erythrocytes. Evidence is accumulating that, in culture systems, in the presence of appropriately selected cocktails of cytokines, growth factors, transferrin (as a source of iron), serum and erythropoietin (as the hormone that drives erythropoiesis), it takes approximately 3 weeks to expand erythroid progenitors, more than 10,000-fold, in a culture volume of 20–30 liters. At the end of erythroid culture there is a mixed population of up to 90 % enucleated reticulocytes, free nuclei and residual nucleated cells. A purer population of reticulocytes is obtained by passing the cells through a standard leucocyte reduction filter used for universal pre-storage leucodepletion in blood processing. Several different types of leucocyte removal filters need to be validated to identify the best practical model that reduces the time taken for cells to pass through validated filters without clogging, damaging, or releasing harmful biological response modifiers The recovery and integrity of the functional reticulocytes following leukoreduction should be continuously monitored to ensure their reliability, consistency and reproducibility. Media volume can be reduced by centrifugation processes to concentrate the culture-generated red cells. Closed autotransfusion systems have been optimized to concentrate the cells at the end of culture [2]. Accordingly, volume reduction during production needs to be fully standardized and harmonized to achieve processing consistency.

From a quality assurance and standardization standpoint, continual quality improvement at every stage of culture process is an essential part of all R&D programs producing therapeutic products. Moreover, any changes in the culture protocols that improve the expansion of erythroid progenitor cells must ensure sufficient therapeutic quantities and quality. This is of particular relevance as donor variability and donors' parameters enormously influence cell growth in culture. This observation is crucial since conventional components are known to be subjected to the donor variation effect as well. These issues remain an essential part of the successful culture process, as the selection of donors whose erythroid progenitors have a greater expansion capacity, is a key parameter of the source material. Consequently, moving towards CRBC products might give as the opportunity to leave RBC storage lesions behind, but will lead to a different kind of donor dependency. The appropriate choice of reproducible and stable expansion media that enable rapid rate of growth also remains to be fully investigated.

4. Trends and targets in RBC expansion using controlled bioreactors

The feasibility studies on using immortalized human erythroid progenitor cell lines for generating large numbers of clinically viable CRBCs, in a robust and reproducible way began in early 2000s [13]. To date, major advances have been made in the fields of immortalization of erythroid adult cell lines [13–18]. Currently, we are witnessing significant breakthroughs. For example: The Bristol erythrocyte adult cell line (BEL-A) can effectively expand indefinitely the proerythroblasts' stage of differentiation. Further, gene editing of these cells in order to create individual blood group knockouts has produced a new diagnostic tool and a multi-blood group knockout that could be used as a new therapeutic option for sickle cell disease patients [19]. Genetic modification of the BEL-A cell line led to the generation of cytokine-independent cells and the production of reticulocytes expressing therapeutic proteins for the treatment of patients with enzyme deficiencies or for use in perfusates that could prime donor organs for transplantation [1]. Initially, it appeared that the HPV16-E6/E7 oncogene could force expression of the transcription factor TaL-1 that is essential for the early haemopoiesis development in cell lines. This proved to be helpful in producing low efficiency functional haemoglobin after differentiation of enucleated RBCs. Another immortalized cell line that was developed by the transduction of c-MYC and BCL-XL into multipotent haematopoietic progenitor cells derived from pluripotent stem cells, showed high rates of enucleation and expression of foetal haemoglobin following injection into NOD/SCID mice [16]. Later, by introducing the HPV16 E6/E7 oncogenes into bone marrow CD34+ cells, the first human immortalized adult erythroid line (BEL-A) was produced. These cells have biochemical and structural features of normal erythropoiesis and survive *in vivo* expressing mainly haemoglobin A. This has provided the first proof of principle for the feasibility of scaling up erythroblast expansion in controlled bioreactors by using immortalized cell lines to generate RBCs that share common metabolic and functional characteristics with adult conventional RBCs [16,14–18].

In this context, further quality profiling of the final products is needed in order to ensure that CRBCs have the potential to replace

short- or long-term unwanted events.

5. Future perspectives

Conceptually CRBCs might become the cornerstone of red cell substitution therapy in transfusion medicine. This is an enormous task and only achievable through collaboration of all research groups involved who we hope will work together and help each other. In the context of facing unresolved challenges, in order to address the risk/benefit balance in relation to safely of patients, a fresh review of the jungle of information is warranted, since so many lessons can be learned from the production, the quality, the legally bound policies and the evidence-based recommendations. Collaborative studies in transparent working rooms, osmotic flows of both ideas and findings, and – most of all – a return of excitement to the research classes are needed for the forward movement of such a difficult project. To conclude, we already know enough about CRBC as a massive, powerful tool, but we still have a long way to go before achieving standard RBC product replacement. Balancing between what we have achieved so far and the uncertainty – that is related to the risk/benefit ratio and the cost-effectiveness – of using CRBCs, might prove to be helpful and informative for moving towards the next level of transfusion therapy. With each step taken and each goal achieved it seems that the questions multiply, but this is a challenge that the scientific community must deal with in order to increase the chance for an even brighter future for transfusion therapy.

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