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Review

Ex vivo generation of transfusable red blood cells from various stem cell sources: A concise revisit of where we are now

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ABSTRACT

Blood transfusion is an essential and irreplaceable part of modern medicine, as a therapeutic modality or additional support to other clinical therapies. Nevertheless, the entire procedure from blood collection to administration, absorbs a significant amount of resources and has a number of problems that need to be addressed. The paucity of donors, the transmission of pathogenic microorganisms and the overall costs of the process have switched the scientific interest to the quest of alternative transfusion methods. The industrial ex vivo production of transfusable red blood cells capable of replacing a unit of packed red blood cells is a very attractive prospect, let alone the idea of a massive production of such a biological material. Various scientific groups, by exploiting erythropoiesis, the stem cells' characteristics and the constantly renewed knowledge in the fields of collection, culture, preservation and expansion of stem cells, have made significant progress towards the realization of such an idea. All three major sources of stem cells, haematopoietic stem/progenitor cells, human embryonic stem cells and induced pluripotent stem cells are thought to be capable of generating adequate amounts of red blood cells. By further studying and refining the in vitro red cell production protocols, it is anticipated that the economic and biotechnological obstacles of the current methods will be overcome in the near future. This manuscript is a brief revisit of their current state of the art, potentials and obstacles that are associated with industrial and clinical application issues.

1. Introduction

Transfusion of red blood cells (RBCs) has become an indispensable therapeutic intervention. It improves the quality of life in patients with chronic anaemia [1] and it can be life-saving in several circumstances [2]. However, the blood supply depends on donations and there is a worldwide shortage of RBC units. Based on WHO's fact sheets, the annual global blood collection is reported to be about 112.5 million units [3]. This number is incapable of fulfilling the annual global needs in blood transfusions which are estimated to be more than 300 million units [4]. Such an imbalance is not just calculated from discrepancies in the demand and supply of blood but also from the variable number of blood donations reported from different countries around the world [5]. Approximately half of the units are derived from high income countries, which accommodate only 15% of the world's population [6].

Apart from that, the age of the transfused patients also differs between countries. In developing countries 65% of blood units are transfused to children under 5 years old, whereas in developed countries the majority of blood supply is distributed to patients over 65 years of age [3]. Although it seems that in western countries transfusion needs are met, it is anticipated that blood supply even in these cases will likely become insufficient by 2050. These calculations are based on the increased life expectancy of the world's population combined with the transfusion demands of advanced western medicine [7].

As far as blood safety is concerned, current technologies can detect the vast majority of transfusion transmitted diseases but the risk has not been eliminated; the presence of the "window period" for the known pathogens along with the emerging pathogens, make the risk real [8].

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Moreover, in most of the developing countries the screening potential is insufficient while blood transfusion may be also complicated with adverse reactions [9]. All these complications add further costs either by increasing the need for new screening tests for new pathogens or by hospitalizations [10,11]. Keeping these concerns in mind, the concept of developing surrogate transfusion products seems an attractive and probably unavoidable option.

One promising solution is the production of transfusion-quality RBCs from human cell sources [12]. This innovative approach poses two major questions that need to be addressed, namely, identification of the most appropriate source of human stem cells and their culture conditions for large scale production of RBCs. Artificial blood has already been used in the laboratory for experimental purposes; however, the clinical use of such a product requires ex vivo generated RBCs functionally equivalent to native RBCs, a heavy technological feat that has not yet been accomplished [13,14].

2. The starting point issue

The main source materials for the production of RBCs in vitro are the haematopoietic stem/progenitor cells (HSPCs), the embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPSCs) [5,12]. These types of stem cells can differentiate into RBCs via culture protocols consisting of three main stages: commitment, expansion and maturation [15,16].

The produced RBCs should be evaluated in vivo for survival, functionality, and safety. The reported studies so far have been performed in mouse models. To ensure survival of the cultured RBCs, the animal should become a cross-species permissive host. This is achieved by inducing a state of immunodeficiency. The most commonly used animal model is the non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse. Recently, novel animal models have been developed such as the chronically anemic SCID, the NOD/LtSz-scid and the IL-2Rγc null (NSG) mice. The latter is a strain of NOD/SCID derived line that is among the most immunodeficient animal models described to date. Overall, the animal models that are used for evaluating the ex vivo generated RBCs should be able to recapitulate the conditions of clinical transfusion settings. Often however, a model is designed in a way that makes it more suitable for addressing a specific experimental question. For example, an anemic mouse is most appropriate to study the oxygen delivery of cultured RBCs, whereas a deeply immunodeficient mouse is better suited for studies that question the survival of transplanted cells [17,18]. Clearly, human trials are far more complicated and to date there is only one report of testing manufactured RBCs on a single human recipient [19].

2.1. Ex vivo generation of RBCs from HSPCs

Haematopoietic stem/progenitor cells (HSPCs) are $CD34^+$ cells that can be isolated from bone marrow (BM), peripheral blood (PB) and cord blood (CB). The ability of those cells to promote ex vivo erythropoiesis has been studied by many research teams. The protocols include addition of specific combinations of growth factors to the cell culture medium in a sequential fashion [20].

Initially, Neildez-Nguyen et al. reported the generation of RBCs from HSPCs isolated from CB. The culture method involved the cytokines Flt3 ligand, thrombopoietin, stem cell factor, erythropoietin, and insulin like growth factor I, added at three sequential culturing steps. The production of erythroid precursors was characterized by low enucleation efficiency (4%) and expression of foetal haemoglobin (HbF). The cells however, could further differentiate into enucleated RBCs in vivo by injection into NOD/SCID mice [21].

Three years later, Giarratana et al. used a similar three step culture protocol that included feeder stromal cells of murine origin (MS-5 cell line) in an attempt to recapitulate the bone marrow milieu. Under these conditions, enucleated RBCs were produced by either CB or PB HSPCs at high rates (up to 100%), which survived normally post infusion in NOD/SCID mice. The amplification was tenfold higher for CB HSCPs (10^6) compared to PB HSPCs (10^5). However, this promising method is not clinically applicable due to the enormous demands in culture area (approximately 150 km^2) and the consequent lack of cost-effectiveness [22].

In 2011 the same group reported the proof of principle for transfusion of ex vivo manufactured RBCs starting from PB $CD34^+$ HSPCs in human. Their survival in vivo was comparable to that of native RBCs (half-life of 26 days versus 28 ± 2 days for the native RBCs). Despite the small number of RBCs produced by this method (equivalent to 2 mL of packed RBCs), the study remains a major step forward since it was the first case of a clinical grade cultured product that was tested successfully in human [19].

Satisfactory RBCs expansion from CB HSPCs has been also shown following co-culture with BM- or CB-derived mesenchymal stem cells. The enucleation levels were approximately 64% [23]. Despite the fact that the feeder cell co-culture system provided high expansion and enucleation rates in cultured cells, it has many drawbacks such as difficulties in isolating pure, non-contaminated RBCs, presence of xenogeneic cell types (when cells of murine origin are used), variability in CD34+ expansion and issues of allogenicity when human MSCs are used.

Undoubtedly, the adaptation of feeder-free protocols could reduce both cost and complexity involved. Towards that direction, many protocols have developed and optimized, with a production level of up to 2×10^8 RBCs in bioreactors [24,25]. Moreover, high-yield results were recapitulated by using an FBS-containing culture medium in a roller bottle culture system. By similar robust expansion processes, it could be feasible to produce over 500 units of RBCs starting from one CB unit (5 million $CD34^+$ cells) [26]. Finally, in an attempt to develop a feeder layer- and FBS-free culture process Poloxamer 188 (a polymer with cytoprotective function against hydrodynamic stress) has been applied, which showed high enucleation rate (95%) and enhanced survival of mature RBCs [27].

What has become clear from those studies was that $CD34^+$ from different sources exhibit different erythropoietic potential [28]. And although PB $CD34^+$ would be an ideal source for manufacturing autologous RBCs (especially in cases with alloimmunization or rare blood groups) the low number of circulating HSPCs (without GSCF mobilization) and their lower expansion potential pose limitations for routine clinical adaptation.

2.2. Ex vivo generated RBCs from ESCs

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts, namely, of mammal embryos at early developmental stage. They were initially isolated from mouse embryos in the early 1980s and have been thereafter, a great tool for basic biology and experimental medicine [29]. ESCs, when kept in culture under certain conditions, have the potential of indefinite self-renewal without differentiation. However, when allowed to differentiate back into a host blastocyst, they maintain the ability to generate cells of all three germ layers, a gold standard feature for defining the pluripotency of a stem cell [30].

ESCs from human sources (hESCs) are generated after in vitro fertilization; the major disadvantage of using these ESCs is the ethical issues that arise by their origin, and hence, limitations of integrating them into clinical applications. hESCs have the advantage of being able to divide approximately 300 times while retaining their normal karyotype, pluripotency and full-length telomeres [31]. Differentiation of ESCs begins when factors that specifically contribute to the maintenance of the stem cell state, are withdrawn. Three basic methods are used to trigger differentiation: (i) formation of three-dimensional colonies, the embryoid bodies (EBs), (ii) co-culturing with stromal feeder cells and (iii) culturing on a layer of extracellular matrix proteins [32–34].

The ability of hESCs to differentiate into haematopoietic cells and thus, to the erythroid cell line, was studied with protocols involving EB

formations and co-culture system [35–37]. The stromal cells used in most experiments were derived from non-human cell lines. The EBs-based systems resulted in erythropoiesis that resembled morphologically the stage of definitive erythropoiesis, except for haemoglobin that was of embryonic or foetal stage [38].

Initially, Lu et al managed to isolate haemangioblasts, namely bipotential precursors of haemopoietic and endothelial cells, which could differentiate in multiple haemopoietic lineages including erythroid lineage. Two limitations were observed, namely the lack of enucleation and the expression of foetal haemoglobin [39]. An improved protocol by the same group led to enucleation of 65% of cells and expression of adult haemoglobin after prolonged culture time in a proportion of cells (15%). By applying this protocol, however, only 10^{10}-10^{11} RBCs were developed, compared to the unit of packed RBCs that contains approximately 2×10^{12} cells [40].

Definite erythropoiesis (enucleation along with adult haemoglobin expression) and robust RBC expansion remain an issue when ESCs have been used as the primary source of cells. According to many studies, culture time lengthening or forcing the expression of transcription factors involved in erythropoiesis, e.g. RUNX1a or HOXB4, would enhance the expression of beta-globin genes [41–44]. Finally, a more efficient, serum- and feeders-free potential system for differentiation has been also validated [45].

2.3. Ex vivo generated RBCs from iPSCs

Generation of induced pluripotent stem cells (iPSCs) was based on the methodology developed by Yamanaka's laboratory in 2006; iPSCs were produced by reprogramming somatic cells back to inner cell mass-like cells through forced experimental expression of a set of four genes (Oct3/4, Sox2, c-Myc and Klf4) [46]. Human iPSCs may derive from any somatic cell and behave similarly to hESCs; they are capable of self-renewal, large-scale expansion and differentiation into all cell types of the three germ layers in vitro. The major advantage of iPSCs over ESCs, however, is that they can be produced from any cell type, allowing thus, selection of donor's phenotype without posing the ethical dilemmas arisen by using ESCs. On the other side, the main disadvantage of iPSCs is that not efficient reprogramming may lead to a mix of fully and partially reprogrammed cells and thus, to a far from perfect technology to pluripotency. Several iPSC generation protocols that involved strategies for avoiding viral transfer of genes have been developed, the most recent and encouraging of which refer to entering key genes into the cell via episomal carriers, synthetic RNA transcripts and through recombinant proteins. Protocols used for ex vivo expansion are similar to those of ESCs and include formation of EBs or use of co-cultures with feeder cells [47–50].

Establishment of iPSCs from dermal fibroblasts of a Bombay blood type individual was first reported in 2009, by applying the Yamanaka's technique [51]. The pluripotent cells had the characteristics of hESCs: they could differentiate into all three haemopoietic cell lines and expressed HbF. In 2010 Lapillonne et al. designed for the first time a two-step cell culture protocol for the direct commitment of foetal and adult fibroblasts-derived human iPSCs to definitive erythropoiesis. Despite success in reprogramming, the amplification and enucleation rates differed significantly compared to those involving ESCs (10% vs 66%). Moreover, haemoglobin synthesis was blocked at the stage of HbF, independently of the origin of the reprogrammed cells [52].

As shown later on, iPSCs can achieve terminal maturation in terms of enucleation in vitro, but complete maturation, in terms of both enucleation and haemoglobin switch happens only in vivo, that is, in an adult haematopoietic microenvironment, following injection and maturation of progenitors into NOD/SCID mice in situ. Thus, the iPSCs can walk all the erythroid way ahead toward full maturation but not under the current in vitro differentiation context. Mature RBCs were kept in circulation for four days and the total cell number generated from 1×10^6 iPSCs was 15 to 28,3 $\times 10^8$ RBCs [53]. To improve enucleation rate, inhibition of specific miRNAs (i.e. miR30 A) was proposed [54].

Recent advances in the scale-up production include:

a) development of a good manufacturing practice (GMP)-compatible, feeder-free and serum-free method that takes advantage of small molecule effectors to specifically promote erythroid differentiation of hPSCs (with a potential to generate 50.000–200.000 erythroid cells from one HSPC) [12,55] and,
b) application of expanded cultures of iPSCs and ESCs in spinner flasks [56]. ESCs seem to be superior to iPSCs in terms of expansion and enucleation rate but still both sources are inferior to the potential of CB and PB $CD34^+$ cells, posing thus limitations for scale up production necessary for clinical applications.

2.4. Immortalized cell lines

Robust and reproducible erythroid precursor immortalization techniques may finally provide efficient numbers of viable and functional RBCs for clinical use in vitro. The first demonstration of the feasibility of using immortalized human erythroid progenitor cell lines as an ex vivo source for producing RBCs came in 2013, by using the HPV16-E6/E7 oncogene and forced expression of the transcription factor TaL-1 that is essential for the early haemopoiesis. Nakamura's laboratory developed cell lines able to produce enucleated RBCs (though at a low efficiency) with functional haemoglobin after differentiation in vitro [57].

An immortalized erythrocyte progenitor cell line was also developed by the transduction of c-MYC and BCL-XL into multipotent haematopoietic progenitor cells derived from pluripotent stem cells. Differentiation was achieved by turning off the overexpression of those factors. The cells expressed foetal haemoglobin and showed high rates of enucleation following injection into NOD/SCID mice [58].

In 2017, Trakarnsanga et al. generated the first human immortalized adult erythroid line (BEL-A) by introducing the HPV16 E6/E7 oncogenes into bone marrow $CD34^+$ cells. BEL-A RBCs had biochemical and structural features of normal erythropoiesis and developmental potential to functional, enucleated reticulocytes that survived in vivo expressing mainly haemoglobin A [59]. This year, the first proof of principle for the feasibility of scaling up erythroblast expansion in controlled bioreactors by using the ImEry cell line was reported. These cells were derived from immortalizing $CD71^+CD235a^+$ erythroblasts isolated from adult PB. The generated RBCs seemed to share common metabolic and functional characteristics with those of adult RBCs [60].

3. Obstacles that need to be overcome

The erythroid cells that are intended to usage in clinical applications must be produced at a large scale and at a terminal differentiation state. It is well established that the culture conditions affect the proliferation potential of the cultured progenitor erythroid cells as well as their enucleation capability. Conditions such as the kind and the concentration of growth factors or the timing of their administration in the three-step culture system mentioned above, seem to have a significant impact on the efficiency of enucleation; these conditions need to be optimized and further defined.

Deep knowledge of peptides and molecules that play an integral role in definite erythropoiesis and their (or counterpart imitators, such as the erythroid macrophage peptide or VCAM-1) supply exogenously could induce enucleation through ligand-receptor interactions [55,61]. Additionally, for an optimization of the differentiation process and stable enucleation in vitro, mifepristone (an antagonist of glycocorticoid action), or factors involved in vesicle trafficking have been applied in the culture systems [62,63]. In the same context, histone acetyltransferases or histone deacetylases involved in chromatin remodeling, and caspases involved in apoptosis could also serve as possible targets for the in vitro modification of the enucleation process. However,

chromatin remodeling factors are non-specific, affect many genomic regions and might be able to either downregulate genes which are vital to cell functions or activate silenced oncogenes. The long-term consequences of using such agents require further studies [64,65].

Beyond culture conditions, usage of feeder cells has the potential to enhance maturation and optimize cell population expansion, but the presence of foreign material sets essential restrictions on clinical applications since (i) the cost is higher due to the huge number of stromal cells required, (ii) the technique is more complicated, and (iii) the stromal cell lines carry the risk of contamination by xenogeneic pathogens [22,23,66]. In addition, the common static culture conditions have failed to reach the required expansion of ex vivo generated RBCs. A major step forward has been materialized with bioreactors that allow three-dimension cell growth and consequent increments in production yields [24,25,56].

It seems that the best way to increase the production of RBCs would be the generation of committed cell lines derived from the ideal stem cell source, that is a cell population able to provide unlimited proliferation. Genetic manipulation of these cell lines is unlikely to be an obstacle to their clinical application, provided that the cells are enucleated. This can be ensured by filtration or by irradiation. Moreover, in order to maintain genetic and epigenetic stability of the cell lines, periodical controls should be performed on their genotype [57–60].

3.1. Functional issues

During the ex vivo production of RBCs, additional controls are required for testing the multiple physical and biochemical factors that characterize the final product. The manufactured RBCs mostly resemble the native RBCs that are generated under stress conditions, since they are slightly macrocytic and express greater amounts of HbF, a fact that clearly affects the oxygen dissociation curve. However, transfusing HbF-containing RBCs may not be contraindicated taking into account the fact that subjects with hereditary persistence of foetal haemoglobin (HPFH) are asymptomatic and moreover, increase in HbF expression is the aim of targeted therapies in patients with haemoglobinopathies, such as sickle cell disease [67,68]. In contrast, the metabolic pathways and the proteome of the produced RBCs show a high degree of similarity to the native RBCs, indicating that the manufactured cells have a closer affinity with their native counterparts at those functional levels [67]. Nevertheless, differences have been reported depending on the stem cell sources used. Another consideration is the high heterogeneity of the expansion and enucleation potentials, as well as of the haemoglobin type that is expressed. Indeed, both hESC- and iPSC-derived cells have low enucleation capability and express mainly embryonic and foetal globin genes. Additionally, the reported yields from these sources are lower in comparison to those reported from HPSCs [69,70].

3.2. Scaling issues

The use of bioreactor systems has enabled scale up culture of HSCs and erythroid cells. The clinical application of RBCs generated ex vivo requires mass production (10^{12} cells/unit) of a safe and functional product at an acceptable cost. The calculated cost ranges from 8,000–15,000 USD if hPSCs serve as a source [25]. Usage of low-cost media and reagents, replacement of culture media with small molecules, omission of the feeder cells and generation of erythropoietic cell lines, may result in a more cost-effective product. Safety issues, such as accidental transmission of harmful agents and immunogenic reactions, may be overcome if the protocols used for the production of ex vivo RBCs comply with the Good Manufacturing Practice (GMP) [45,55,71].

3.3. Clinical applications

Manufactured RBCs from selected donors are currently applied as reagent RBCs for antibody identification in alloimmunized patients, and as a vehicle for drug delivery [13]. The design of clinical trials for testing transfusion of ex vivo generated RBCs should be careful since there are still several inconsistencies in the procedures and the protocols. In vitro generated RBCs should be compatible with ABO and Rhesus antigens (a sum of eight antigens), suggesting that eight different erythroid cell lines would be sufficient to supply the transfusion demands of the majority of world's population [59]. It is anticipated that ten clones of human iPSCs representing the most common RBC phenotype combinations could meet the need for transfusion of 99.43% of alloimmunized patients [52]. Alternatively, RBCs can be produced by using healthy O Rh negative or Bombay blood type donors or by applying innovative techniques which omit blood group antigens (enzymatic cleavage or antigen masking), providing thus universal RBCs for all recipients [72–74]. It is a fact that this innovative medical product has stimulated scientific interest, and as research continues in this direction, it is anticipated that RBCs production ex vivo will definitely become a significant tool in the field of transfusion therapy in the near future. The first clinical application of in vitro generated RBCs would be in rare blood groups and chronic transfusion dependent patients.

4. Conclusions

At the beginning of the 21 st century, the deeper knowledge of haematopoiesis in general and of erythropoiesis in particular, as well as the constant evolution in the field of scientific and technological advances, seem to make feasible the in vitro generation of RBCs for transfusion in the near future. However, many improvements are necessary, in order to achieve this goal. As proof-of-principle, ex vivo production of RBCs with the functional characteristics of native RBCs has been achieved. It is a matter of cost-efficient scaling to allow this technology to reach clinical applications, and in this leap forward, industrial development will have a major input.

Conflicts of interest

None.

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