INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

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Expansion of hematopoietic stem cells from umbilical cord blood in roller bottles

Tesis que presenta

Hera Andrade Zaldívar

Para obtener el grado de

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Director de la Tesis:

Dr. Antonio De León Rodríguez

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Créditos Institucionales

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que se desarrolló bajo la dirección de

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El presente trabajo fue realizado en el laboratorio de Biotecnología del Departamento de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, bajo la dirección del Dr. Antonio de León Rodríguez- Durante la realización de este trabajo se contó con el apoyo económico del proyecto Fondos Sectoriales CONACyT-SALUD 13993 y de manera personal se contó con la beca 103403 del CONACyT.

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Content

RESUMEN

Los transplantes de progenitores hematopoyéticos de sangre de cordón umbilical son empleados en una variedad de padecimientos hematológicos y oncológicos cada vez con más frecuencia. En la mayoría de los casos, el número de progenitores hematopoyéticos encontrados en una unidad de cordón umbilical es insuficiente para transplantar a pacientes adultos. El objetivo de este trabajo es ofrecer una perspectiva sobre las opciones para derrotar ésta limitante y proponer una alternativa de cultivo *in vitro* para incrementar los progenitores hematopoyéticos de sangre de cordón umbilical. En primer lugar se presenta una revisión concerniente a la expansión *in vitro* de los progenitores hematopoyéticos y posteriormente se ofrece una investigación original sobre el uso de un sistema de frascos giratorios para su cultivo y expansión en ambientes con y sin dióxido de carbono. Los resultados de la expansión de células hematopoyéticas en frascos giratorios muestran que éste sistema es una alternativa operacional y económicamente viable para la expansión de progenitores hematopoyéticos, permitiendo una expansión de entre 15 y 20 veces en la mayoría de las condiciones probadas. Además, se reporta el uso del medio de cultivo L-15 para el cultivo o expansión de progenitores, que mejora la expansión y que podría simplificar el cultivo de células hematopoyéticas en gran escala.

Palabras clave: Células madre, cultivo celular, frascos giratorios, transplante de células madre, cultivos libres de CO_2.

ABSTRACT

Umbilical cord blood stem cell transplants are a current treatment in several hematological and cancer conditions. In most cases the hematopoietic stem cell content in a cord blood unit is low and this limits their use in adult patients. The objective of this work is to offer a perspective about the options to overcome this issue and propose a culture alternative to increase the hematopoietic stem cells *in vitro*. We present a review on the *in vitro* expansion of hematopoietic stem cells alternatives and an original investigation based on the use of a roller bottle system to culture hematopoietic stem cells in both CO_2 and CO_2 free atmosphere. In most conditions tested, the expansion system allowed a 15-20 fold expansion of hematopoietic progenitors, demonstrating that the roller bottle system is functional economically and operationally. We report a novel use of the culture media L-15 to expand hematopoietic progenitors, using this media may simplify the large scale culture systems for stem cells.

Keywords: Stem cells, cell culture, roller bottles, stem cell transplant, CO_2 free atmosphere

INTRODUCTION

1.1 Hematopoiesis and hematopoietic stem cells

The blood system is formed by several types of cells with specific and vital functions. However, blood cells have a limited life span, e.g. a few hours for granulocytes, 120 days for erythrocytes and some years for memory B cells [1].

The human body renovates $1x10^{12}$ blood cells daily through a complex process known as hematopoiesis [2]. After born, this process takes place in the bone marrow and consists in the generation of all blood cell types from a single common progenitor named hematopoietic stem cell (HSC).

There are 2 main lineages of blood cells, myeloid and lymphoid, both derived from the most primitive HSC, known as long term HSC, passing through the short term HSC and the multipotent progenitors and several committed cell stages. A general view of hematopoisis is shown in Fig 1.

The hematopoiesis is a vital function of HSC, however only approximately 1 in 10000 bone marrow cells are HSC [3], therefore there must be a process of self renewal to maintain a constant population of HSC that guarantee the adequate supply of blood cells throughout the life cycle of any organism. Therefore, HSC must convey two main characteristics: self renew and be capable of generating all further differentiated blood cell types. This functional definition is necessary because, despite the scientific effort, there is not a general agreement in the cell surface markers that may identify accurately the HSC [4]. The most accepted combination of such markers is shown in table 1.

In the bone marrow, the microenvironment is formed by cells, including macrophages, reticular cells, adipocytes and osteoblasts that together with the extracellular matrix and some cytokines and growth factors provide the adequate atmosphere for the hematopoiesis and/or self renewal. However, the molecular mencanisms guiding these processes are still poorly understood.

Table 1. Antigenic characterization of human HSC

CD 34^{+}	$Thy1^{+}$
C-$kit^{-/low}$	$CD59^{+}$
lin^{-}	$CD38^{low/-}$

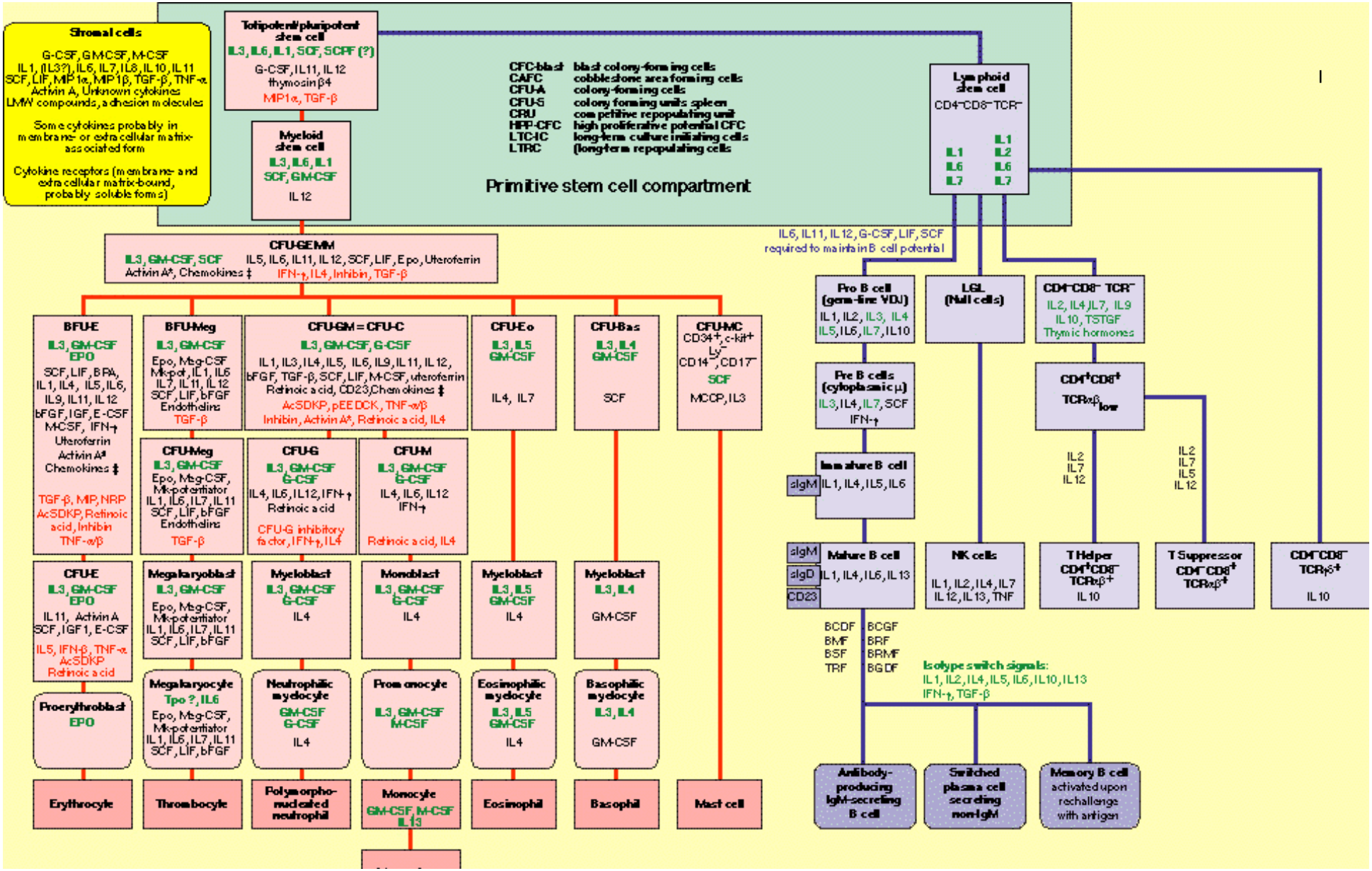

Fig1. Summarized representation of hematopoiesis. The earlier progenitors (in darker edge boxes) pass through several stages to form the final functional mature cells (gray boxes) guided mainly by the cytokines indicated in the boxes (green mainly and black prssumibly. Took from Horst Ibelgaufts. (1995) Dictionary of Cytokines. Vch Pub. HSC: hematopoietic stem cell; CFU, colony forming unit; BFU, Burst forming unit; Meg: Megacariocytic; E, erythroid; Eo, eosinophyl ; G, granulocytic M, monocytic; GM: granulocytic-monocytic; Bas, basophyl; PMN, polimorpho nuclear; CM, mastoid; IL, Interleukyn; SCF, stem cell factor; G-CSF, Granulocyte stimulating factor; GM-CSF, Granulocyte-monocyte stimulating factor.

1.2 Hematopoietic stem cell transplantation

Last century, several experiments demonstrated that after transplantation HSC have the ability of reconstitute permanently all the blood cell lineages in lethally irradiated recipients [5, 6]. This has permitted to successfully transplant HSC in several diseases, including hematological malignancies, autoimmune disorders, metabolic alterations and even solid tumors [7]. Transplantation consists in the injection of HSC into the bloodstream of the recipient, after which HSC engraft in the bone marrow to continue their normal functions in the new organism.

The HSC may be taken from several sources such as bone marrow, peripheral blood or umbilical cord blood, from the same recipient before the ablation treatment (autologous transplant) or from a compatible healthy donor (allogenic transplantation).

Bone marrow collection requires a painful needle aspiration of the donor. Peripheral blood aphaeresis involves the administration of G-CSF to mobilize the HSC to the circulating blood. Both procedures are time consuming and painful for the donor, and after the transplant they may have a high risk for the Graft versus host disease (GVHD). Umbilical cord blood was considered a waste material of births, but nowadays it may be frozen and stored in banks. The use of HSC from umbilical cord blood highly reduces the GVHD chances [8]. However the number of HSC in UCB is small, being the main drawback of UCB usage in transplantation.

Next chapter presents a review of the efforts made to increase the HSC number available for transplantation, which is what concern this work as well.

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1.3 Expansion of human hematopoietic stem cells for transplantation: trends and perspectives. (MINIREVIEW)

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Abstract

Umbilical cord blood transplantation is clinically limited by its low progenitor cell content. *Ex vivo* expansion has become an alternative to increase the cell dose available for transplants. Expansion has been evaluated in several ways such as static cultures combining growth factors or mimicking the natural microenvironment using co-culture systems. However, static cultures have a small volume capacity and therefore large-scale expansion has been addressed using bioreactors. These and other biotechnological approaches for expanding hematopoietic progenitors and their utility to study several aspects of hematopoietic stem cell biology are discussed here.

Key words: 2D-culture, 3D-culture, human cells, leukemia, transplant

Introduction

Blood tissue transplantation is not limited to traditional blood transfusions containing mature blood cells. It is also possible to renovate the entire blood system transplanting hematopoietic stem cells (HSC) to allow a full recovery of the hematopoietic system in patients with different hematological disorders such as aplastic anemia and leukemia. This approach opened a wide range of clinical applications for progenitor blood cells transplantation including gene therapy [9] and the generation of specific mature cell types [10,11].

HSC are self-renewing multipotent cells able to produce all blood cell lineages. They are found in the bone marrow and they can migrate to the circulating blood only with the proper stimulation [12]. Umbilical cord blood (UCB) is other major source of HSC, which has been successfully used for transplantation since 1989 [13]. The possibility of creating UCB banks is a reality [14]; therefore UCB is increasingly gaining attention as a reliable source of HSC for transplantation, and it is currently an accepted therapy for several diseases. Bone marrow and peripheral blood stem cells autologous transplantation reduces the risk of immunological rejection and are preferred as therapeutic approaches. But, various reports showed that UCB has several advantages over the other sources of HSC when allogenic transplantation is needed, e.g. accessibility and non-invasive collection procedure, a lower chance of host *versus* graft disease and more flexibility for multi-lineage differentiation [15]. The main drawback in UCB transplantation is the low quantity of HSC, since the content per UCB unit ranges between 0.4 and 1.0 $x10^9$ total mononuclear cells (MNC), whereas the dose currently recommended ranges from $2.0x10^7$ to $2.5x10^7$ MNC/kg. A dose lower than $1.5x10^7$ MNC/kg showed poor results [6], thus restricting UCB transplantation to pediatric patients in most of the cases. Since the biology of HSC and their microenvironment are not totally understood [16], it has not been easy to overcome this issue. *Ex vivo* expansion of HSC from UCB and other sources became an alternative to increase the cell-dose available for transplants and to further research on HSC. There is evidence that even if short-term expansion may modify HSC properties, it is strongly probable that the engraftment characteristics remain unaltered [17].

HSC require an adequate microenvironment to keep their stem properties. In bone marrow the microenvironment is very complex, HSC are surrounded by bone matrix and different cells including fibroblast, adipose, macrophage and endothelial cells which produce various cytokines

and growth factors; these signaling molecules induce HSC to differentiate or to remain in the stem state maintaining a balance in hematopoiesis.

In vitro HSC culture requires a suitable microenvironment, for that reason, different culture media, growth factors and supplements have been tested (Table 1). Most investigations have used Iscove´s modified Dulbecco's Medium (IMDM) plus animal or human serum and combinations of cytokines. Alternatively, serum-free and animal product-free media have been developed to avoid immunological issues affecting transplantation. Different cytokine cocktails aim the proliferation of undifferentiated HSC and the maintenance of their engraftment capacity. The optimal combination and concentration of growth factors to preserve the stem state has not been yet established [18, 19], but a mixture of stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (Flt-3L) is enough to support the expansion of HSC. However, no correlation has been found between the concentration of cytokines and the expansion of $CD34^+$ cells [10]. Another option to mimic the stromal niche is co-culture HSC with accessory cells [20].

Table 1. Comparison of different expansion protocols for human UCB hematopoietic stem cells using various cytokine combinations.

Cytokine cocktail	FBS	Expansion of CD 34^+ cells at day 7 (fold)	Reference
SCF, GM-CSF, IL-3, TPO, Flt-3L	20 %	11.21±9.27	[10]
SCF, GM-CSF, IL-3, TPO	20 %	10.18±8.64	[10]
SCF, GM-CSF , IL-3, TPO, IL-6	20 %	9.87±8.57	[10]
SCF, Flt-3L , TPO, IL-3, IL-6	20 %	3.2	[47]
SCF , IL-6 , IL-3	20 %	2.44	[34]
SCF, TPO, Flt-3L, IL-3, G-CSF, IL-6	0%	27	[48]
SCF, GM-CSF, IL-3, TPO, Flt-3L	0 %	25.11±13.50	[10]
SCF, GM-CSF, IL-3, TPO	0 %	24.98±13.66	[10]
SCF, GM-CSF , IL-3, TPO, IL-6	0 %	24.56±13.37	[10]
G-CSF, IL-6, EPO	0%	6.36±0.33	[49]

FBS: fetal bovine serum

In vitro HSC culture should be a closed bioprocess to avoid the contamination, keep HSC undifferentiated and achieve a fold expansion enough to transplant adult patients. Different methods to expand HSC attempt the highest expansion with the lowest manipulation. Expansion in traditional static cultures e.g. T-flasks, culture bags and multi-well plates are not suitable for HSC, since these cells are likely to clump at the bottom and there is not proper nutrient and oxygen distribution. Moreover, it has been reported that primitive cells cultured under static conditions lose their unique stem features [21]. Suspension cultures and other platforms appeared as alternatives, e.g. perfusion chambers [22,23], and stirred tanks [24,25,26], showing advantages over static cultures. Reviews on approaches to expand HSC [27,28] and clinical trials [29,30] have been published elsewhere. Therefore, we have focused this review on more recent biotechnological approaches aiming to increase the HSC content for transplantation.

Three-dimensional static cultures

The first cultures of UCB-HSC were performed in classic static culture dishes. However, static cultures do not provide the proper three-dimensional (3D) environment. Therefore, new strategies have been designed. The microenvironment of HSC has been mimicked with 3D scaffolds. In one study UCB-HSC in a serum-free/cytokine-free medium were expanded on a commercial 3D carbon matrix (Cytomatrix) covered with fibronectin [31]. The culture system consisted of multi-well plates with or without the matrix. Since culture medium was serum and cytokine free, cells cultured without the matrix did not show expansion at all. Expanded cells showed engraftment capacity in the sub lethally irradiated severe combined immuno-deficient and non-obese diabetic (NOD/SCID) murine model. Similarly, the utility of 3D-scaffolds over 2D-cultures was demonstrated in a study using a fibronectin-immobilized 3D polyethylene terephthalate (PET) synthetic matrix, where UCB-$CD34^+$ cells were cultured for 10 days in

serum-free media yielding a 100-fold time expansion. Long term culture initiating cells (LTC-IC) which are responsible for long term engraftment presented a 47-fold expansion, in addition, the expanded cells allowed reconstitution of hematopoiesis in the NOD/SCID murine model [32].

Ceramic foams of Al_2O_3 and apatite have been used to provide a 3D structure similar to bone [33]. Total nucleated cells from bone marrow and mobilized peripheral blood ($1x10^6$ cells/ml) were seeded on the foams in 6 well-plates. The static culture was carried out in IMDM supplemented with human AB serum and cytokines. After 12-27 days of culture, three morphologies and cell clusters covered the ceramic surface, supporting the multipotent capacity of the cells, although confirmation on the phenotype or the quantity of cells produced was not provided. Static systems with 3D scaffolds have demonstrated the expansion of HSC keeping the repopulating ability. However, the recuperation of the cells requires mechanical or enzymatic detachment, which may damage the cells. These cultures ranged from 0.1 to 2 ml and the scale-up has not been performed, thus it is uncertain whether they will be useful for clinical applications.

Some groups have established co-cultures to provide a proper 3D microenvironment for HSC. Current work is focused on the creation of murine cell lines able to support UCB-HSC expansion [34]. The main drawback of xenogenic feeder cells is the risk of pathogens leading to infection and immunological reactions. Several studies tried to overcome this issue. For example, Fujimoto *et al.* attained a 194-fold expansion of UCB-MNC in 2 ml cultures using microencapsulated mouse or human feeder cells [35]. Feeder cells obtained from human bone marrow have been used to expand UCB-HSC in 5 ml cultures with SCF, TPO, Flt-3L plus human serum [36]. Adherent cells obtained from UCB cultures have been used as feeder layers to support the expansion of HSC [37, 38]. However, the engraftment capacity and the immunological reactions after transplantation need to be further researched. Xie *et al.*

established an indirect co-culture system using retroviral transduced human mesenchymal stem cells expressing Flt-3L and TPO, and adding complementary cytokines to culture UCB-$CD34^+$ cells. In a serum free co-culture of 7 days the MNC expansion was almost twice the expansion achieved using only cytokines. The colony forming unit of granulocyte, erythrocyte, monocyte and megakaryocyte (CFU-GEMM) showed a 13.55±4.15 fold expansion while using only cytokines it was 3.23±1.28. However, $CD34^+$ and total CFU did not show a significant difference. Expanded cells also showed a bigger increase in the LTC-IC and similar engraftment to uncultured cells in the NOD/SCID murine model [39]. The feeder layer systems have achieved expansion, but these approaches require preparing mono-layers previous to the culture, making the process slower. Besides, extra manipulation is needed to harvest the expanded HSC. The use of monolayers in large-scale cultures has not been evaluated either. This, together with the immunological issues makes unknown whether these methods may be clinically relevant.

Static 3D approaches seem to expand HSC more efficiently than traditional 2D static cultures. But the use of inert supports or stromal cells involves additional steps to recuperate the cells - e.g. trypsin treatment- that may damage or reduce the number of cells.

Leaving aside the 3D support, Madlambayan *et al*. designed a static bioprocess consisting of two gas permeable culture bags separated by a magnetic system to eliminate undesired cells from the culture [40]. The multi-step process included seeding of cells, a first incubation of four days, separation of the fraction of interest, centrifugation for growth media renewal, and additional sub-cultivation for four days. With this device the total cell, $CD34^+$, CFU, and LTC-IC fold expansion achieved 24.6±3.6, 30.8±7.2, 31.3 ± 5.8 and 32.6±7.5 folds, respectively. It was demonstrated positive engraftment in the NOD/SCID murine model. This process was used for volumes up to 24 ml with little manipulation of the cells and it may have clinical application if automation is achieved.

Dynamic cultures

Despite static cultures have shown expansion of HSC, the scaling-up represents a major problem because more volume means less oxygen flow and less nutrient availability in the system. Several dynamic models that incorporate gas flow have been used to overcome this problem. Diverse bioreactors with specific characteristics (Table 2) have been designed for HSC expansion since the 90's, but the latest designs have served as well to study the HSC biology.

Table 2. Recent approaches for human HSC expansion.

System	Cells	Volume (ml)	Media and Growth Factors	Fold Expansion	Engraftment	Reference
RWV 0-6 rpm 8 days	$2x10^5$ UCB-MNC/ml	33	IMDM, 10% FBS, 10% horse serum, 5.33 ng/ml IL-3, 16 ng/ml SCF, 3.33 ng/ml G-CSF, 2.13 ng/ml GM-CSF, 7.47 ng/ml Flt-3L and 7.47 ng/ml TPO	MNC: 435±87.6 $CD34^+$: 32.7±15.6 CFU-GM: 21.7± 4.9	Not tested	[36]
Spinner flask 30 rpm 7 days	$1x10^6$ mBM $CD34^+$/ml	30	IMDM, 20% FBS, 50 ng/ml SCF, 5 ng/ml IL-3 and 10 ng/ml IL-6	MNC: 1.27 $CD34^+$: 5.43 CFU:10.60	Not tested	[34]
DIDECO Pluricell 30 rpm 12 days	$2x10^4$ fresh UCB-$CD34^+$/ml	38	Unknown with Flt-3L, TPO, IL-3, SCF and human plasma	MNC: 230.4±91.5 $CD34^+$: 21.0±11.9 CFU: ND	Yes	[35]
Static 3D matrix Fibronectin covered 14 days	$2.5x10^5$ UCB-$CD34^+$/ml	1	StemSpan (serum free) Cytokine free	MNC:ND $CD34^+$: 3 CFU-GM: 2.6	Yes	[23]
Static 3D matrix Fibronectin-immobilized PET 10 days	$1.0x10^3$ UCB-$CD34^+$/ml	0.1	StemSpan (serum free) 100 ng/ml SCF, 100 ng/ml Filt-3L, 50 ng/ml TPO, 20 ng/ml IL-3	MNC: ND $CD34^+$: 100 LTC-IC: 47	Yes	[24]
Static disposable bags 8 days	$1.0x10^3$ UCB-$CD34^+$/ml	2.3-24.5	StemSpan (serum free) 100 ng/mL SCF, 100 ng/mL Flt-3L, 50 ng/mL TPO, 1 □g/mL low-density lipoproteins	MNC: 24.6±3.6 $CD34^+$: 30.8±7.2 CFU: 31.3 ± 5.8	Yes	[32]
Stirred tank bioreactor 75 rpm 7 days	$5x10^5$ UCB-MNC/ml	120	IMDM, 10% FBS, 1 ng/ml IL-3, 5 ng/ml SCF, 1 ng/ml GM-CSF and 3 U/ml EPO	MNC:1.27 $CD34^+$: ND CFU: 7	Not tested	[16]

ND: Non determined

1. Stirred systems

Bioreactors have been useful to expand HSC as well as to study their *in vitro* biology [15-19, 41]. For instance, Qunliag *et al*. developed a comparative gene expression analysis of UCB-

HSC grown in static and spinner flasks cultures [42]. The differences in gene expression between the two systems were evaluated using cDNA micro-arrays and semi-quantitative PCR. They found 11 of 103 genes over-expressed in static culture which are involved in oxidative stress response and DNA repair. Genes over-expressed included Superoxide dismutase 1 (SOD-1), glutathione S-transferase theta 1 (GSTT-1), excision repair cross-complementing rodent repair deficiency, complementation group 1 and 3 (ERCC-1 and ERCC-3), tumor necrosis factor receptor superfamily member 1 (BTNFRSF1B), BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP-3), glucose phosphate isomerase (GPI), and transcription factor forkhead box O1A (FOXO). These elements probably constitute the HSC response to the low-oxygen and the deprived-nutrient environment provided by the static culture, which cause gradient concentration of nutrients, growth factors, metabolites and poor gas flow. Another interesting finding was the over-expression of delta-like homolog 1 (DLK-1) in static culture. It is thought that DLK-1 blocks the differentiation of hematopoietic primitive cells. Differential expression of genes and proteins in diverse growth conditions leads to elucidate the active pathways in HSC, which could help to improve HSC expansion. However, this experiment only reported 1.27, 5.43 and 10.60-fold expansions of MNC, $CD34^+$ cells, and CFU respectively, showing low potential for clinical applications.

The DIDECO Pluricell system (Patent pending) is a commercially available bioreactor consisting of a 175 cm^2 polystyrene expansion chamber, equipped with a series of filters and bags to permit the injection of media, gas, cells and outlets for sampling and collection of the expanded cells. Culture media and components are certified as serum-free. This appliance has been used to expand fresh and cryopreserved human $CD34^+$-UCB cells for 12 days with an injection of fresh media on day 7 of culture. FACS analysis determined that most of the progenitors expanded were of myeloid and megakaryocytic lineage [43]. In a 38 ml culture of fresh $CD34^+$-

UCB samples, 249.1±49.5 and 33.0±14.3-fold expansions of MNC and $CD34^+$ cells were achieved, respectively. This system generated an average $1.75x10^8$ MNC, just enough to transplant a 6 kg patient with $2.9x10^7$ MNC/kg. The expanded cells showed significant engraftment in the NOD/SCID murine model, however human trials must be performed. This system showed a high MNC expansion, and the use of certified reagents for the culture may settle it for clinical trials.

2. Rotating Wall Vessels

Stirred tanks and perfusion chambers produce shear stress, which may cause mechanical damage to HSC. One approach to maintain homogeneous environment with low stress, is the use of rotating wall vessels (RWV). Yang Liu *et al.* [44] designed a RWV bioreactor to culture total MNC from UCB (Figure 1).

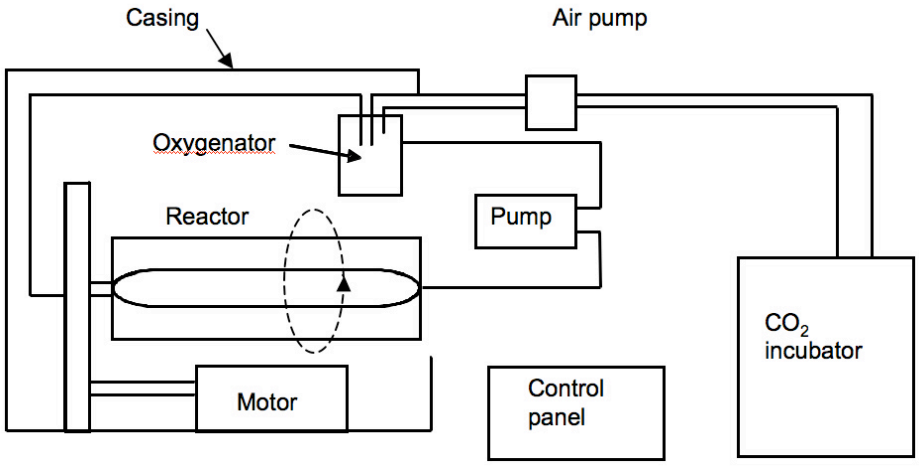

Figure 1. Schematic representation of a rotating wall vessel bioreactor. Adapted from Liu *et al*. [36].

Cells were cultured at an increasing rotating speed from 0 to 6 rpm. The RWV achieved large expansion of MNC, however the engraftment capacity of the expanded cells was not evaluated.

It will be necessary to research whether the cells are affected at the molecular level under these culture conditions. They suggested that the multi-step RWV bioreactor could expand a single cord blood to reach $1.2x10^9$ MNC, enough to transplant an 80 kg patient (with the minimal amount of $1.5X10^7$ CMN/kg). However, the cell dose is below the standards defined in the current guidelines for transplantation [6].

The National Aeronautics and Space Administration (NASA) developed two RWV bioreactors for tissue mass culture [45]. The slow turn lateral vessel (STLV) bioreactor has been used to culture several kinds of cells both in Earth and in space. The STLV (Fig 2a) was operated at 15-30 rpm in Earth and slower in space allowing a free-fall state, reducing the shear stress. The high aspect ratio vessel (HARV) bioreactor (Fig 2b) has a similar design, but the rotating speed can be slower than STLV. Both systems were used to culture human embryonic stem cells (hESC), showing that STLV reduced the aggregation of hESC and they attained a 4-fold increase in productivity respect to the Petri dish cultures [46]. The NASA-RWV systems have been used to study the effects of microgravity on murine HSC and evaluating the hematopoietic homeostasis during long space expeditions [47]. These RWV bioreactors could be used for human HSC expansion for transplantation.

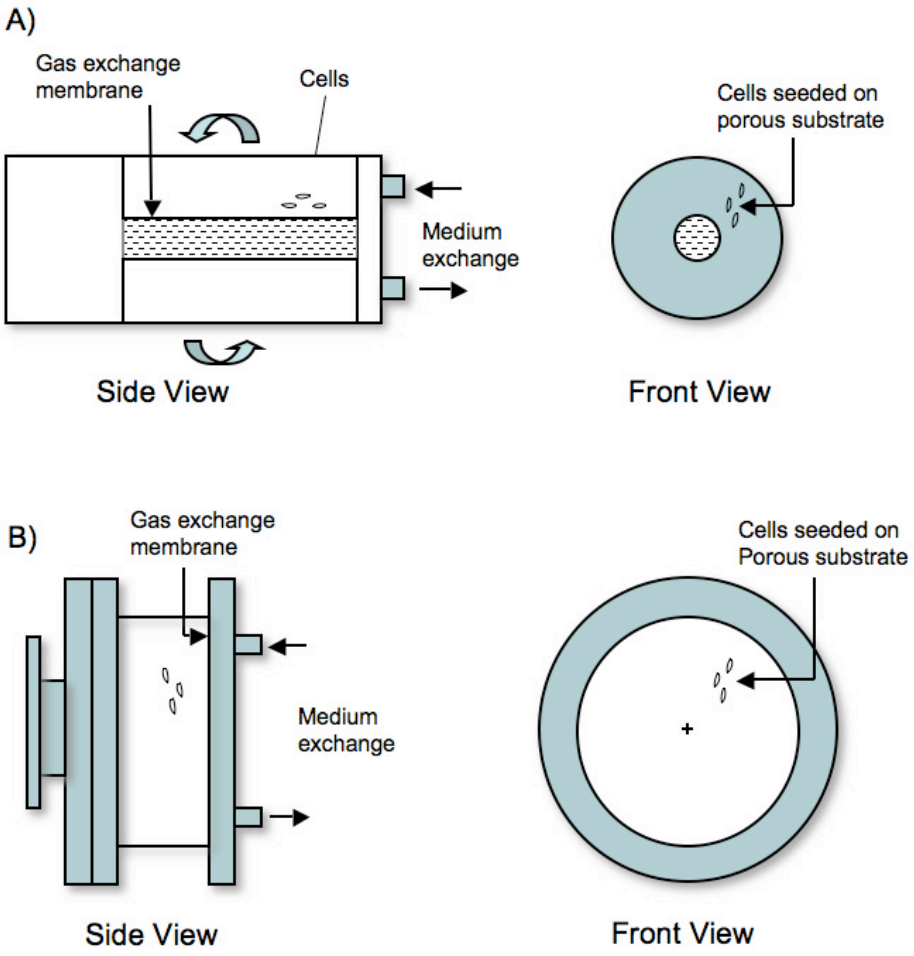

Figure 2. NASA-RWV bioreactors. A) Slow turn lateral vessel. B) High aspect ratio vessel, adapted from [38].

3. Novel systems

There are other strategies to design bioreactors minimizing shear strees. For example, Zellwerk GmbH-HiPer-Gruppe has developed a novel rotating bed perfusion (RBP) system equipped with ceramic carrier discs arranged horizontally (Fig 3a). Discs rotate slowly allowing the cells to alternate between medium and air [48]. The RBP system has been used to culture osteoblasts and other kind of adherent cells, including stem cells, thus it may be a promising approach to expand HSC. Cesco Bioengineering Co., Ltd developed a novel disposable packed bed contractile (DPBC) bioreactor that provides low shear stress because it is not agitated and it

does not need sparging air, resembling an artificial lung. The DPBC (Fig 3b) has been successfully used to produce various proteins and viruses and it is suitable for adherent and non adherent cell cultures including embryonic stem cells [49]. The DPBC bioreactor could be used to expand HSC, but the recuperation of the cells from the bioreactor could be problematic.

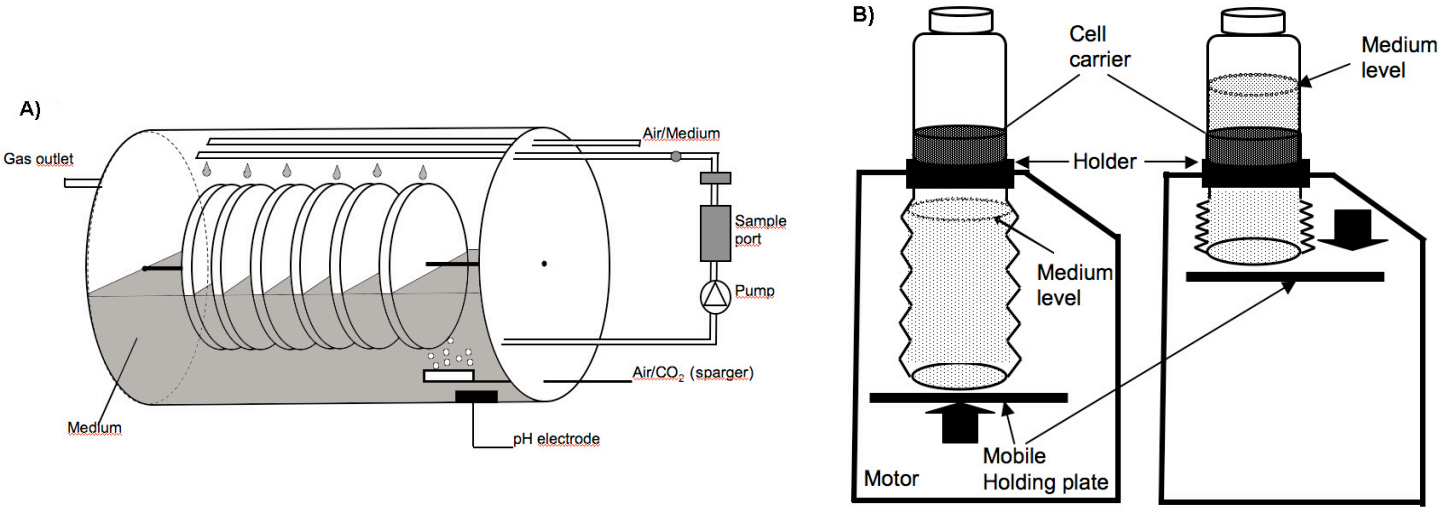

Figure 3. A) Rotating bed perfusion system, Zellwerk GmbH-HiPer-Gruppe. B) Disposable packed bed contractil bioreactor, Cesco Bioengineering Co., adapted from [40] and [41].

Concluding Remarks

There is a growing range of clinical applications for UCB-HSC and a lack of efficient tools to expand them. The characterization of the optimal conditions for *in vitro* culture of HSC is still a challenge [50]. The optimal combination and quantity of cytokines, use of serum, time of culture, initial cell density, enrichment of $CD34^+$ cells, use of stroma, and other factors are not fully determined. The TPO, Flt-3L and SCF mix is used in most static and dynamic cultures for HSC expansion. In most cases, the time of culture is from 1 to 2 weeks, but no higher expansion is shown in the longer cultures and the optimum time of culture is uncertain. The maximum MNC expansion achieved in stirred systems was up to 100-fold, whereas the low-rate agitated RWV systems attained 435-fold. The largest volume used in bioreactors was 120 ml [16] and larger

scale-up has not been evaluated yet. Despite the few reports on the use of bioreactors for HSC expansion, it has been demonstrated that they are generally better platforms than static 2D cultures to expand MNC from UCB.

Considering the problems derived from long term culture of stem cells such as phenotypic changes and chromosomal alterations [51], it is necessary to establish characterization methods for expanded HSC to assure that cells maintain the same features and they are safe for transplantation. The characterization of cells must include the alterations by epigenetic factors, since they lead to aging and differentiation [52, 53, 54]. Transcriptomic and proteomic studies could be helpful for this purpose. The elucidation of mechanisms governing self-renewal and differentiation of HSC is needed to control the *in vitro* expansion.

Results from pilot clinical trials of transplants using expanded UCB-HSC have shown no adverse effects in the patients. However, more clinical trials must be conducted using expanded UCB-HSC for guarantying the safety.

Acknowledgements

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2. OBJECTIVES

General objective

- To evaluate the feasibility of roller bottle cultures for the *in vitro* expansion of umbilical cord blood hematopoietic stem cells.

Specific Objectives

1. Assess and compare the *in vitro* expansion of HSC in static and roller bottle cultures of total mononuclear cells from UCB using different culture media.
2. Assess and compare the *in vitro* expansion of HSC in static and roller bottle cultures of selected $CD34^+$ cells from UCB

3. EXPANSION OF HEMATOPOIETIC PROGENITORS

3.1 Expansion of human hematopoietic cells from umbilical cord blood using roller bottles in CO_2 and CO_2 free atmosphere

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Stem cells and development (2010). PMID: 20825280

SUMMARY

In this work, we evaluated the expansion of human hematopoietic stem cells from umbilical cord blood in roller bottles (RB). The Iscove´s modified Dulbecco's medium, the Stem Pro 34-SFM medium, and the L-15 Leibovitz's medium for cultures in CO_2-free atmosphere were assessed. At day 5 of culture, total colony forming unit expansions of 14.44±3.74, 11.20±6.37 and 17.25±3.65 folds were attained, respectively. The expansion reached using L-15 medium in RB was around 10 times higher than the achieved in the static control cultures. To our knowledge, this is the first report of cultures in CO_2 free atmosphere to expand cord blood human hematopoietic stem cells and it opens a new branch of possibilities for culturing and clinical applications.

HIGHLIGHTS

- Roller Bottle cultures suit hematopoietic stem cell expansion
- CO_2 free atmosphere cultures improve human hematopoietic stem cell expansion

INTRODUCTION

Human hematopoietic stem cells (HHSC) can be obtained from several sources such as bone marrow (BM), mobilized peripheral blood (MPB) and umbilical cord blood (UCB) [1]. BM transplants have been a life saving tool for more than 40 years in the treatment of malignant and non-malignant diseases such as leukemias and aplastic anemia [2], but the collection procedure is invasive and matching donors are not always available. Umbilical cord blood is an approach with high potential for HHSC transplantation. The advantages of UCB are a non-invasive collection procedure, the possibility of establishing cord blood banks, easier finding of compatible donors and a lower risk of host versus graft disease [3,4], but the main drawback is the small amount of HHSC that can be obtained from UCB (0.4- 1.0 $X10^9$ total mononuclear cells).

A wide range of possible therapeutic applications for HHSC is being studied; therefore strategies to exploit the UCB potential, such as *in vitro* expansion are increasingly needed. *In vitro* expansion of HHSC has been proven viable and safe, since expanded cells can be transplanted to patients without risk [5,6]. Therefore, several studies have been focused on the culture and expansion of HHSC. The proposed strategies include from 2-dimensional and three-dimensional static cultures to different types of bioreactors including *airlift*, perfusion chambers, stirred tanks, spinner flasks and rotating wall vessels with promising results [7-9]. Unlike most animal cell cultures where cell-products are harvested and cells are disposable, the major interest of HHSC culture are the cells themselves. HHSC require adequate oxygen and nutrient flow, which may be achieved with agitated bioreactors, but since they grow in suspension they

are sensitive to shear stress and the mechanisms to sparge oxygen can cause cell damage. A lower agitation rate could minimize shear stress [10]. Roller Bottles (RB) are a simple strategy for culturing adherent and suspended cells, they can be operated without specialized training, they are easily scalable for clinical purposes and they involve very low capital investment [11]. RB have been used for a long time to culture animal cells [12], and are now being used to culture various types of cells including hybridoma [11]. RB provide a good choice to culture suspension cells that are sensitive to shear stress because they can be operated at very low agitation rates.

In this work, we evaluated the utility of roller bottles to expand HHSC from UCB comparing the non-static and static cultures in different culture media with recombinant cytokines.

MATERIALS AND METHODS

UCB collection and processing

UCB samples from full-term deliveries were kindly provided by local hospitals, according to their ethic committee's guidelines. The UCB-MNC separation procedure has been described elsewhere [16]. Briefly, 40-80 ml blood samples were centrifuged 30 min at 850 *g*, then 5-7 ml of white inter-phase cells and plasma were transferred into a 15 ml falcon tube and diluted 1:2 with Phosphate buffered saline pH 7.2 (PBS). Cells were transferred to 15 ml Falcon tubes containing 7 ml of Ficoll-Paque Plus (Pharmacia) at room temperature, and centrifuged 30 min at 1250 *g.* MNC ring was aspirated and transferred to a clean tube, washed twice with PBS and resuspended in 1 ml of IMDM.

Culture Media

Three culture media were tested: Iscove´s modified Dulbecco's medium (IMDM, Gibco), Leibovitz's L-15 (L-15, Gibco) and Stem-Pro® 34SFM (Stem Pro, Gibco). IMDM and L-15 were supplemented with 10% fetal bovine serum (Gibco). Media were supplemented with human recombinant cytokines (Peprotech): 2 ng/ml interleukin-3 (IL 3), 5 ng/ml interleukin-6 (IL 6), 5 ng/ml stem cell factor (SCF), 5 ng/ml granulocyte colony stimulating factor (G-CSF), 5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), 5 ng/ml flt3 ligand (Flt-3), and 3 U/ml erythropoietin (EPO).

Roller bottles cell culture

RB batch cultures were started with 0.5 $x10^6$ MNC/ml into 500 ml glass roller bottles (Wheaton) containing up to 25 ml of culture medium. Cultures were maintained for 14 days at 37°C in an incubator (Shel lab) at 5% CO_2 atmosphere for the IMDM and Stem Prom cultures, whereas experiments in L-15 medium were maintained in a CO_2-free incubator (Shel lab). Roller bottles were set in a bottle bench top roller (Wheaton) at 1 rpm. 24-well plates with 1 ml of the respective culture medium were used as control. The RB caps were tightly closed for the L-15 cultures, whereas they remained slightly open for the cultures requiring CO_2. Sampling was performed taking out the RB from the incubator, removing the caps and washing out the RB walls with the same culture media to recover attached cells, then 0.3 ml were taken for the colony forming cell assay and MNC counting. Medium was not added, removed or altered in any way during these cultures.

Fed-batch cultures were set in IMDM as described above, 25 ml of complete fresh medium were added at the day 3 or 7. Therefore, the final volume was twice of the initial volume of the cultures.

Colony-forming cell assay and mononuclear cell counting

Number of colony-forming cell (CFC) was determined from methylcellulose-based semisolid cultures (Metho Culture; StemCell Technologies) containing per ml: 50 ng SCF, 10 ng IL-3, 10 ng GM-CSF, and 3 U. Plates were inoculated with 10 000 to 40 000 cell/ml and incubated for 14 days at 37°C and 5% CO2. Hematopoietic colonies were classified as described previously [20]. MNC concentration and viability were determined by cell counting with the Trypan Blue exclusion method using a hemacytometer.

Data processing

Error bars in all graphs represent the standard error of the mean.

RESULTS

Total cell expansion

The total cell growth kinetics for the cultures of HHSC under static and dynamic conditions is illustrated in fig 1. Panel A shows a typical static-culture using IMDM. For this sample, the maximum cell concentration was $1.22x10^6$ cell/ml at day 13 and $0.063x10^6$ cell/ml at day 10 for the static and RB culture respectively. Fig 1B shows the cell growth of one representative sample cultured in Stem Pro. After the initial death phase of 5 days, the maximum cell concentration was reached at day 10 of culture, with 1.07x $x10^6$ and 2.83x $x10^6$ cell/ml for the static and RB cultures respectively. The cultures in Stem Pro

reached higher total cell numbers, which can be confirmed comparing panel B to panel A, which is the same sample cultured in IMDM. Fig 1C shows the total cell growth of a culture in L-15. Nearly all controls showed a death phase of only 3 days, but most RB cultures pass through this phase starting the total cell growth from the beginning of the experiment. In this case, the maximum cell concentration, achieved at day 10 of culture, was $1.23x10^6$ and $1.21x10^6$ cell/ml for the static and RB culture respectively.

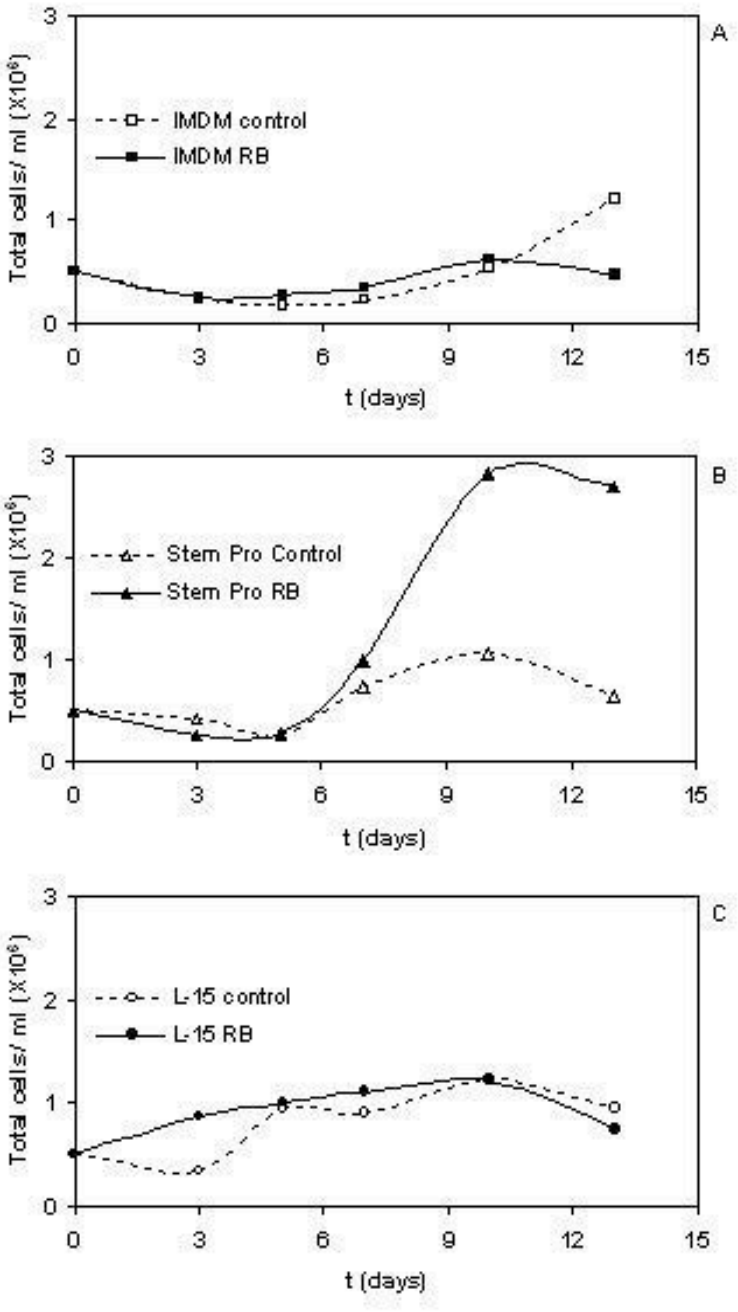

Figure 1. Typical growth kinetics of human hematopoietic cells from umbilical cord blood in static and roller bottles. A) Cultures in IMDM B) cultures in Stem Pro medium C) Cultures in L-15 medium.

Fig. 2 illustrates the total cell fold expansion in our cultures. Panel A shows the IMDM cultures. Some cultures showed a death phase of 5 days; subsequently most control cultures showed continuous growth reaching a maximum cell concentration between days 5 and 13 ranging from 2.44 to 5.30-folds, with a mean of 3.30±0.97 folds at day 13 of culture. However, in the RB cultures each sample showed a unique behavior indicating even a decrease on total cells during the experiment ranging from 0.47 to 5.40 fold-expansion in different days of culture, with a mean of 1.85±0.51 folds at day 13 of culture. Both static and RB cultures showed the same tendency, at days, 3, 5 and 7 the growth was increasing equivalently in both systems, and by days 10 and 13 we observed the same trend to increase total cell numbers but in the static controls at a major extent.

Fig 2B shows the total cell fold expansion in the Stem Pro cultures. The maximum expansion ranged from 0.5 to 2.14 folds in the controls and from 0.35 to 5.66 in RB on different days for each sample. For this medium, the growth was comparable for both systems from days 0 to 7, with a slightly higher growth for the static cultures. For days 10 and 13 we observed a huge variability on the total cell numbers. The mean maximum fold expansion, achieved at day 13 of culture, was 1.18±0.23 and 1.17±1.06 for the static and RB cultures respectively.

Total cell fold expansion achieved in L-15 cultures is shown in Fig. 2C. The maximum expansion attained ranged from 0.67 to 3.3 and from 0.75 to 2.50 folds for the static and RB cultures respectively, in different days depending on the samples. The growth in both static and RB cultures provided comparable results of total cells for the same samples. For this medium we observed the

maximum growth between days and 7 and 10 of culture, but the total increase on cells was lower than for the other two media. The maximum fold expansion in L-15 cultures was 1.35±0.49 and 1.31±0.39 for the static and RB cultures respectively. Comparing panels A and C, the control IMDM cultures showed a higher average total cell expansion than the L-15 controls at days 10 and 13, but for days 3 to 7, both systems showed approximately the same fold expansion in IMDM and L-15 media. The Stem Pro cultures had a lower total cell expansion from day 3 to day 13 in both static and RB systems compared to the IMDM cultures.

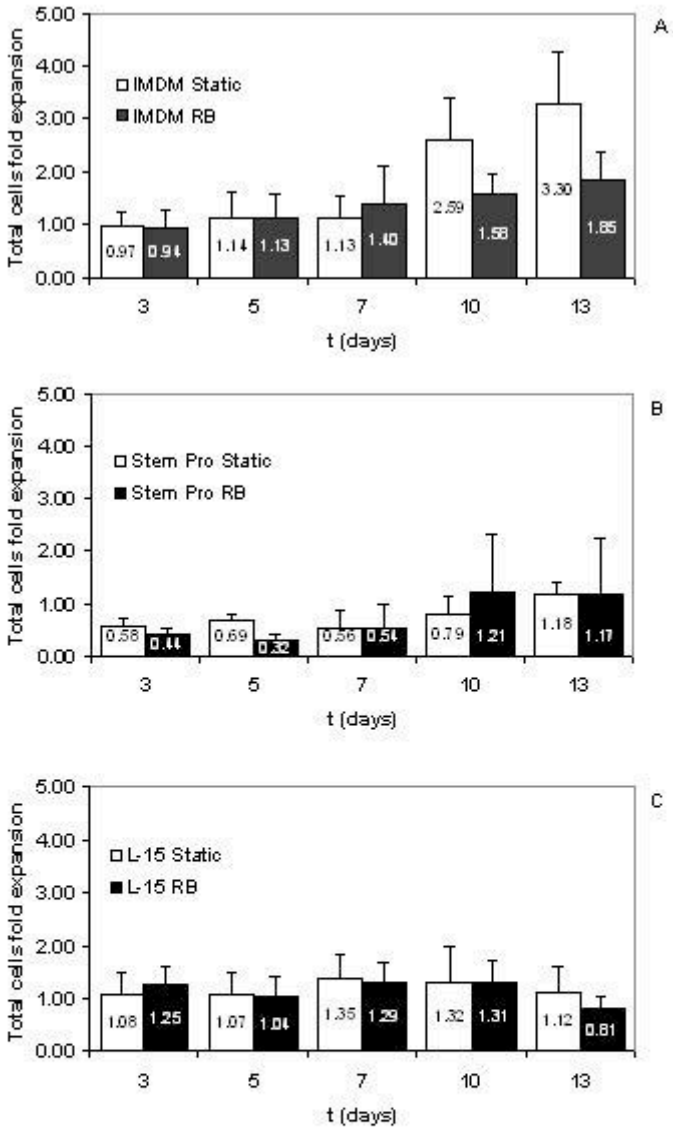

Figure 2. Maximum total cell expansion in static and roller bottles. A) Cultures in IMDM. B) Cultures in Stem Pro medium C) Cultures in L-15 medium.

Expansion of total hematopoietic progenitors

The progenitor expansion achieved in static and RB cultures in IMDM is shown in figure 3A. For RB cultures, we found a mean colony forming cell (CFC) fold expansion of 14.44±3.74, 16.87±5.30 and 9.16±4.15 at days 5, 10 and 13 respectively; meanwhile the static controls achieved 10.10±2.58, 14.02±3.21 and 10.96±4.10 respectively at the same days. Progenitor expansion was observed in this work as early as day 3 of culture (data not shown) despite the fact that the cultures showed a total cell decrease. Confirming these, at day 5, even when the total cell numbers was still lower than the initial, the progenitors in RB cultures were already expanded.

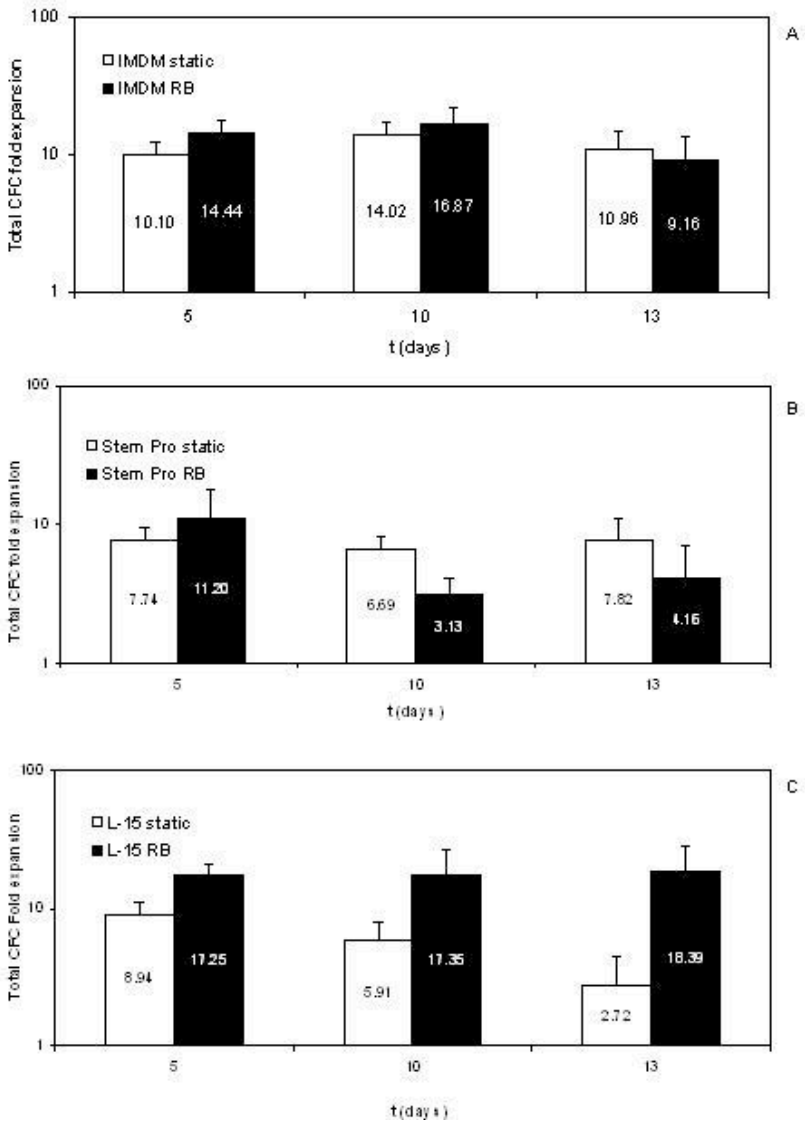

Figure 3. Total Progenitor Expansion in static and roller bottles. A) Cultures in IMDM. B) Cultures in Stem Pro medium C) Cultures in L-15 medium.

The mean CFC fold expansion for the samples cultured in Stem Pro is shown in figure 3B; in these cultures we achieved an 11.20±6.37 CFC-fold expansion on day 5 in RB cultures, whereas for the static control the expansion was 7.74±1.67. However, for days 10 and 13, the progenitor expansion achieved in RB was almost a half of the static controls. Fig. 3C shows the total progenitor fold expansion attained in L-15 cultures of different samples. On day 5, the mean total progenitor fold expansion in RB was 17.25±3.65, on day 10 it was 17.35±8.81 and on day 13 it remained the same with an 18.39±9.49 fold expansion.

Fed-batch cultures using roller bottles

We also performed 15 days fed-batch cultures in IMDM, providing the cultures with fresh media and cytokines in order to support a longer expansion. Fig 4 shows the mean progenitor fold expansion achieved in two fed-batch cultures of the same sample. Feeding the culture on day 3, resulted in a progenitor fold expansion increase from 17.84 to 27.47 on day 5, and from 0.28 to 19.99 on day 13 but on day 10 it decreased from 24.43 to 18.17. Feeding the culture on day 7 did not increase total progenitor expansion in any of the days tested. The static control cultures showed the same behavior than the RB (data not shown).

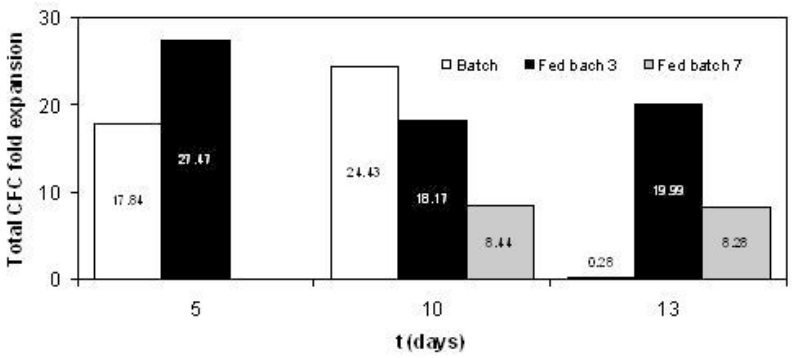

Figure 4. Progenitor expansion in fed batch culture of mononuclear stem cells.

DISCUSSION

RB-cultures in all media tested allowed a 15-20 total progenitor fold expansion on day 5 of culture. Progenitor expansion may be affected by the individual variation since samples were not related at all. IMDM is an improved synthetic medium created for rapidly multiplying cell cultures. This medium, supplemented with different amounts of FBS and cytokines has been widely used for the culture and expansion of HHSC [7, 10, 16, 20] showing good results for CFC expansion in most experiments. We used IMDM for our RB system, obtaining expansion results comparable to other cell culture strategies (table 1). Our RB mimic the expansion found in the controls, but they have the advantage of being able to support larger volumes.

Table 1. Comparison of different protocols for the expansion of HHSC.

System/media	Time of culture (d)	Mean Total cell fold expansion	CFC fold expansion	Volume (ml)	Reference
Roller Bottles IMDM	10	1.40 ±0.69	16.87±5.30	25	This work
Roller Bottles Stem Pro	5	0.32±0.07	11.20±6.37	25	This work
Roller Bottle L-15	5	1.04±0.39	17.25±3.65	25	This work
Rotating wall vessels IMDM	8	435.5±87.6	21.7±4.9	33	Liu et al [10]
Spinner flask IMDM	7	1.27	10.60	30	Qunliang et al [17]
Spinner flask IMDM	7	0.8	1.2	30	Yang et al [18]
DIDECO Pluricell System Med-A	12	230.4±91.5	Not determined	38	Astori et al [19]

We also tested two other media: Stem Pro and L-15. Stem Pro is a serum free medium created to support the growth of $CD34^+$ hematopoietic cells and total cells in static cultures of bone marrow $CD34^+$ hematopoietic cells [21]. The use

of Stem Pro may reduce the risk of immune reactions and infection due to serum [13]. However, Stem Pro-RB cultures showed reduced total cell growth and progenitor expansion in most of our experiments. L-15 medium is buffered by phosphates and free-base amino acids instead of the sodium bicarbonate system used by IMDM. It can be used in non-sealed containers as our RB. We found that IMDM and L-15 cultures reached similar progenitor expansion on day 5, but L-15 permitted the longest total CFC expansion, it remained around 17 times, for all the days tested; therefore it allows a higher and longer CFC expansion than IMDM.

Commercial spinner flasks bioreactors are available to expand HHSC; for example the Dideco Pluricell system with a limit volume of 38 ml achieved a maximum mean expansion of 230 fold in MNC and 21 fold in $CD34^+$ cells. However, shear force influences the development of different types of cells. High agitation rates generally cause a decrease in cell viability and cell concentration [22-24]. A lower agitation rate could minimize shear stress. Low speed agitation in some cases does not affect the suspension growing cells [23]. Even a shear stress of 5 dyn/cm^2 has increased the hematopoietic colony-forming potential of embryonic stem cells [25]. Thus changing to low shear stress conditions could be useful for the selection of HHSC during the cultures. For instance, rotating wall vessel bioreactors have been used up to 6 rpm to culture HHSC, achieving 21.7±4.9 fold progenitor expansion [15]. It is important to develop new methodologies to culture HHSC which are easy to perform, safe and do not promote progenitor cells differentiation.

This is the first report on expansion of HHSC-UCB in roller bottles. We showed that roller bottles are suitable for the expansion of hematopoietic progenitors

since they had a slightly higher total cell expansion than the static cultures, and they allowed progenitor expansion to a greater extent. We used 1 ml control cultures because it has been demonstrated that T-flasks with larger volumes do not show significant cell expansion [15]. We attained total cell expansions comparable to those reported using Spinner flask or stirred bioreactors [16-18], but below the cell expansion attained in Pluricel system or other RB-like devices [10, 19]. RB can be an alternative to the use of culture bags, where up 31-fold CFC expansion has been attained, but the reduced volume and the nutrient rechange and oxygen made difficult the scaling up [14].

In this work, we demonstrated that Roller Bottle short-term UCB-MNC cultures allow progenitor expansion up to 18.39 times in L-15 medium. Roller Bottles are suitable to culture MNC from human umbilical cord blood in all media tested in this work *e.g.* L-15, Stem Pro and IMDM. We demonstrated that L-15 medium is a good choice to culture HHSC and it does not require CO_2 control. Roller bottles without CO_2 atmosphere are simple to operate, have low requirements of cytokines, and favor HHSC expansion. The L-15 RB cultures would be easily scalable, and therefore they could have a great potential for clinical applications. Nevertheless, expanded progenitors must be evaluated for safety, engraftment and utility in transplants.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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3.2 Expansion of $CD34^+$ cells from umbilical cord blood in roller bottles

Hera Andrade Zaldívar and Antonio De León-Rodríguez

Summary

Supplemented IMDM roller bottle cultures of $CD34^+$ cells from umbilical cord blood were established aiming hematopoietic progenitor expansion. After 5 days of culture we obtained a mean 16.84±2.82 total CFU fold expansion, which was not sustained for longer periods. The efficacy of roller bottles for culturing $CD34^+$ cells and expanding hematopoietic progenitors in IMDM was demonstrated, encouraging the use of this culture system for other experiments and may be for future clinical applications.

Introduction

Umbilical cord blood (UCB) human hematopoietic stem cells (HHSC) are currently used for transplants after mieloablative processes and some other medical conditions. Despite their proved effectiveness, there are some limitations due mainly to the low content of stem cells in the UCB samples. The surface markers of early hematopoietic progenitors are still in discussion, but $CD34^+$ cells transplants have been successfully used for the total recovery after mieloablative processes [1]. However, the success of transplants is related to the cell dose and the minimum effective number of transplanted cells is $5X10^5$ cells/Kg [2-4], which reduces the possible transplants to low weight patients.

Recently, we showed that a 17.25±3.65 fold expansion of total mononuclear cells from UCB is possible culturing them in a Roller Bottle system [5]. We

wanted to research further the expansion of selected $CD34^+$ cells from UCB in the same system.

Materials and Methods

General methods for UCB collection and processing, colony assays and cell counting have been already published [5].

$CD34^+$ cells Selection

We used the EasySep® Kit (StemCell Technologies) for positive selection according to manufacturer directions. Briefly, $4.38X10^5$-$1.13X10^6$ cells were centrifuged for 5 min at 2100g, supernatant was discarded and cells were washed with Phosphate buffered saline (PBS), then centrifuged at 2100g for 5 min and re-suspended in 200 µl of PBS containing 2% of fetal bovine serum (FBS) in a round bottom tube. 10 µl of *Cocktail Positive Selection Easy Sep* ™ for $CD34^+$ cells, were added and the mixture was incubated 15 min at room temperature (RT). Five µl of nanoparticules *Easy Sep* ™ were added and the mixture incubated 10 min at RT. Two ml de 2% FBS PBS were added and the tube was incubated inside a magnet for 5 minutes, supernatant was discarded without separating the tube from the magnet and the procedure was repeated from the addition of 2 ml of 2% FBS PBS 4 more times. The cells remaining inside the tube after the washes are $CD34^+$ and were used for our experiments.

Culture Media

Iscove´s modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and human recombinant cytokines (Peprotech): 2

ng/ml interleukin-3 (IL 3), 5 ng/ml interleukin-6 (IL 6), 5 ng/ml stem cell factor (SCF), 5 ng/ml granulocyte colony stimulating factor (G-CSF), 5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), 5 ng/ml flt3 ligand (Flt-3), and 3 U/ml erythropoietin (EPO).

Roller bottles cell culture

RB batch cultures were started with $0.25x10^5$ to $0.5\ x10^5$ MNC/ml into 500 ml glass roller bottles (Wheaton) containing up to 20 ml of culture medium. Cultures were maintained for 14 days at 37°C in an incubator (Shel lab) at 5% CO_2 atmosphere. Roller bottles were set in a bottle bench top roller (Wheaton) at 1 rpm. 24-well plates with 1 ml of the respective culture medium were used as control. The RB caps were slightly open for the cultures. Sampling was performed taking out the RB from the incubator, removing the caps and washing out the RB walls with the same culture media to recover attached cells, then 0.3 ml were taken for the colony forming cell assay and MNC counting. Medium was not added, removed or altered in any way during these cultures.

Results

CD34+ enrichment

Average 45±3.2 ml UCB samples were collected; the average total mononuclear cells content was $1.07x10^8 \pm 0.14x10^8$. With the positive selection EasySep® kit an average of $8.80x10^5 \pm 2.75x10^5$ $CD34^+$ cells were recovered, which represents the 0.82±0.26% of the mononuclear cells.

Total cell expansion

Five samples were cultured in IMDM. Fig. 1 shows the typical growth kinetics observed in the $CD34^+$ cells cultures. For this sample the maximum total cell number was attained at day 13 ($0.80X10^6$ and $0.95X10^6$ for the control and Roller bottle cultures respectively). The culture started with 50,000 cells and ended up with 16 to 19 times the initial cell number.

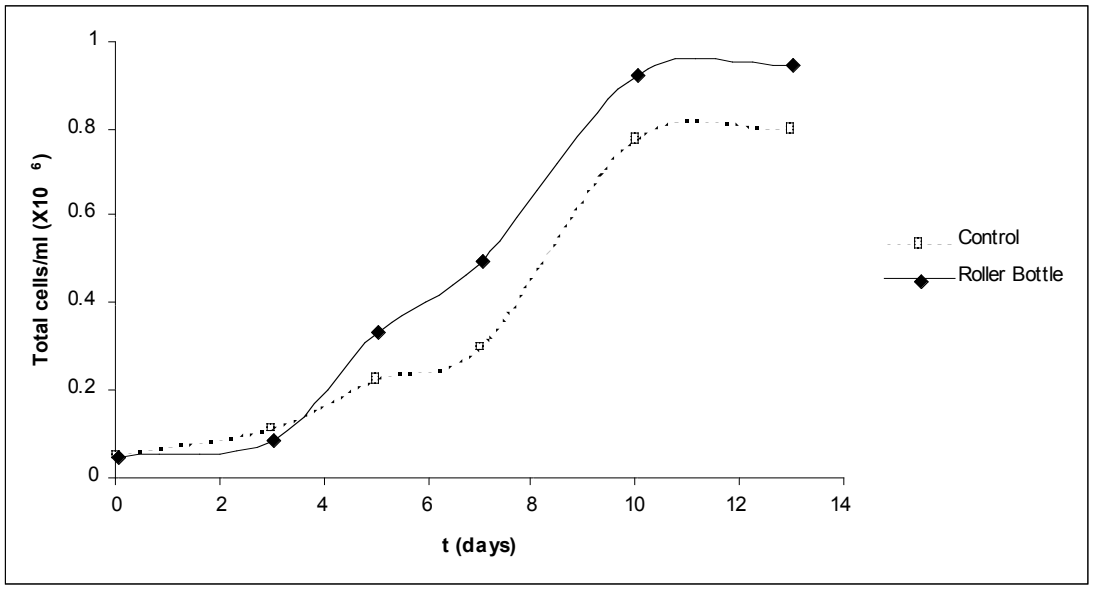

Fig. 1. Typical total cell growth kinetics in static and roller bottle cultures of umbilical cord blood $CD34^+$ cells.

The average total cell fold expansion is shown in Fig. 2, Cells started to multiply from the beginning of the culture and continued the expansion until day 10 of the experiment in both the control and the roller bottle conditions. The maximum cell concentration was attained between days 7 and 10 for the control and between days 10 and 13 for the RB cultures. The ranges of maximum total cell fold expansion were from 2.24 to 54.80 for the controls and from 4.00 to 32.94 for the roller bottles.

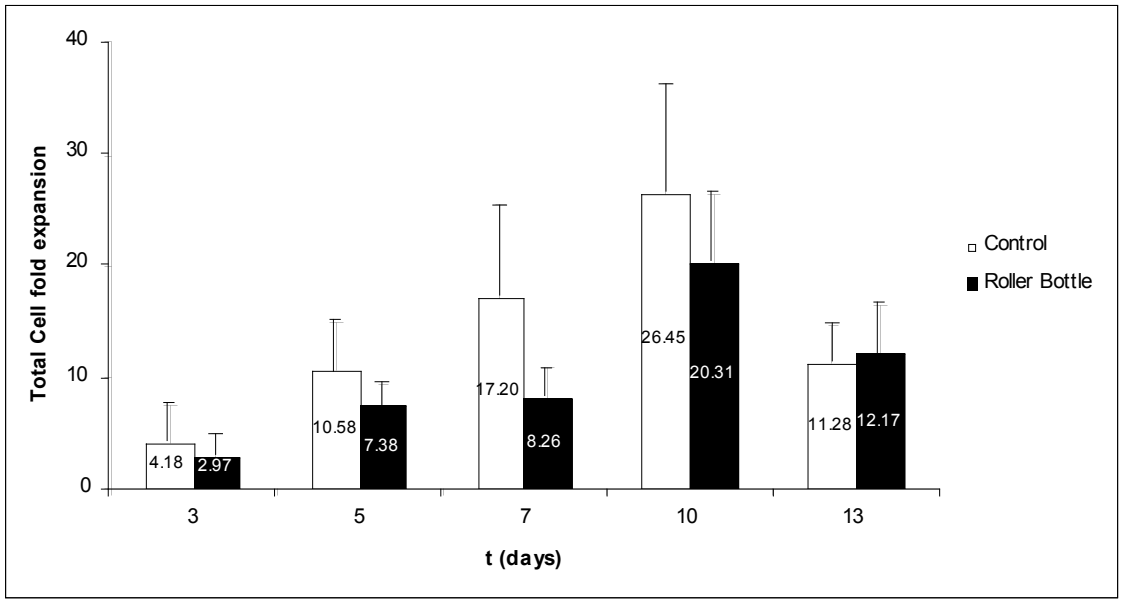

Fig. 2. Average maximum total cell expansion in static and roller bottle cultures of umbilical cord blood CD34$^+$ cells.

Total hematopoietic progenitor expansion

The average total progenitor expansion achieved in 5 cultures of UCB HSC in IMDM media is shown in Fig. 3. On day 5, total CFU increased 17.28±4.47 and 16.94±2.82 folds for the control and the roller bottle cultures respectively. However, for day 10 the CFU fold expansion decreased to 3.00±2.76 for the control and 2.43±1.89 folds for the roller bottle cultures. On day 13 the fold expansion was very difficult to evaluate since we were unable to count the progenitor colonies in three of the five samples cultured in the clonogenic assay.

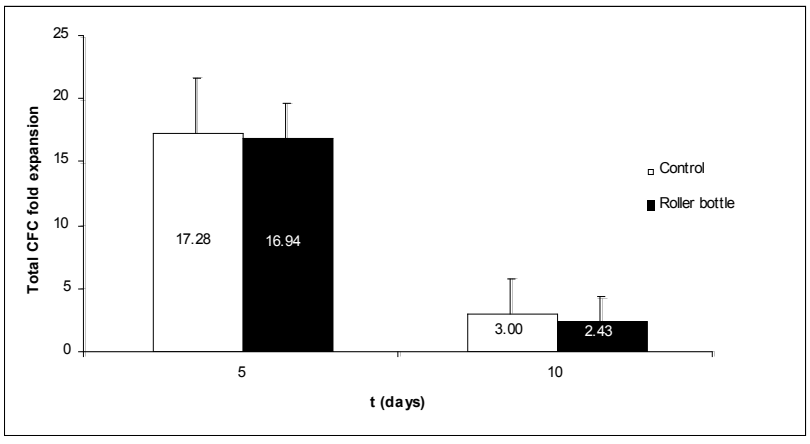

Fig. 3. Total Progenitor Expansion in static and roller bottle cultures of umbilical cord blood CD34$^+$ cells.

Discussion

In the cultures started with $CD34^+$ cells, with only 5 days of culture the number of total cells increased 10 and 7 folds for the control and RB conditions respectively. In the cultures started with $0.5X10^5$ $CD34^+$ cells/ml, the total number of cells per ml reached ranged from $1.13x10^5$ to $2.74X10^6$ for the controls and from $2.00x10^5$ to $1.67x10^6$ for the roller bottle cultures. This numbers are very close to the obtained previously in cultures started with total mononuclear cells instead of selected $CD34^+$ cells [5]. In total cell numbers, our cultures achieved very similar results to other publications, independently from the inoculums size (Table 1).

Table 1. Expansion of Hematopoietic progenitors in UCB $CD34^+$ cells cultures.

System	Volume (ml)	Media	Maximum total cell proliferation		Maximum Progenitor expansion		Reference
			Folds	Day	Folds	Day	
Teflon bags / 14 days $0.25X10^5$ cells/ml	50	CellGRO, 20ng/ml IL3, 100ng/ml SCF and TPO, 300ng/ml Flt3	17±7	6	14±9	6	[6]
24 well plates/ 30 days $0.5X10^5$ cells/ml	1	Myeloculth, 12.5%FBS, 12.5% horse serum, 10ng/ml SF, IL3, IL6, GM-CSF, G-CSF and 3U/ml EPO	500	25	10.45	30	[7]
24 well plates/ 14 days $2X10^5$ cells/ml	1	IMDM, 130ng/ml SCF, (ng/ml IL3, 20ng/ml IL6, 13ng/ml G-CSF, 50 ng/ml Flt3, 0.4U/ml EPO	12.2±4	14	7.6±4.1	7	[8]
4 well plates/ 6 days $0.5X10^5$ cells/ml	1	IMDM, 25% FBS, 50ng/ml SCF, IL3 and IL6, 100ng/ml Flt3	18.19±10.05	6	12.50±14.51	6	[9]
		Stem Pro, 25% FBS, 50ng/ml SCF, IL3 and IL6, 100ng/ml Flt3	16.71±7.23	6	13.60±8.77	6	
Static culture/ 7 days $0.25X10^5$ cells/ml	NP	IMDM, 10% FBS, 500 IU/ml IL1, 10ng/ml SCF, IL3 and GM-CSF	18.23±4.73	7	12.81±9.40	7	[10]
24 well plates/ 25 days $0.1X10^5$ cells/ml	1	IMDM, 10% FBS, 8ng/ml IL3, 3ng/ml SCF, 10ng/ml GM CSF.	60	10	30	25	[11]
24 well plates/ 14 days 0.25 to $0.5x10^5$	1	IMDM, 10% FBS, 2 ng/ml IL 3, 5 ng/ml IL 6, 5 ng/ml SCF, 5 ng/ml G-CSF, 5 ng/ml GM-CSF, 5 ng/ml Flt-3, 3 U/ml EPO.	26.45±9.89	10	17.28±4.47	5	This work
Roller Bottles/ 14 days 0.25 to $0.5x10^5$	9-20	IMDM, 10% FBS, 2 ng/ml IL 3, 5 ng/ml IL 6, 5 ng/ml SCF, 5 ng/ml G-CSF, 5 ng/ml GM-CSF, 5 ng/ml Flt-3, 3 U/ml EPO.	20.31±6.18	10	16.84±2.82	5	This work

We evaluated total progenitor expansion since day 5 of culture, the fold expansions achieved in control and roller bottle cultures were very close, but the RB cultures were performed with 9-20 times more volume, so the total number of CFU was increased as well. The RB cultures of UCB $CD34^+$ cells achieved a maximum progenitor expansion comparable to other published work (Table 1), with the advantage that RB are easily scalable to larger volumes.

Recent work assessed the total progenitor expansion in cultures of UCB total mononuclear cells in a RB system [5]. At day 5 of culture the total progenitor expansion was reported as 10.10±2.58-fold for the control and 14.44±3.74-fold for the RB cultures. Starting with UCB $CD34^+$ selected cells, at day 5 of culture, we obtained a total progenitor expansion of 17.28±4.47-fold for the control and 16.84±2.82-fold for the RB cultures in the same culture system. This data confirm the RB potential for the culture of hematopoietic stem cells. We also performed preliminary experiments in a serum free media, achieving up to 20-fold expansion of total progenitors (data not shown).

By day 10, a decrease of the total progenitor expansion was observed in both the control and the RB cultures, compared to day 5 of culture. In previous RB cultures, started with total mononuclear cells, the progenitor expansion on day 10 was around 2.5 fold greater than for day 5, and in static controls around 4 fold greater [5]. However, in cultures started with $CD34^+$ selected cells, the increase in total cells numbers is almost 10 times more on day 10, than starting with mononuclear cells. The rapidly increasing cell number may be affecting the "stemness" of the progenitor cells by the accumulation of growth factors [12] or the scarceness of nutrients and oxygen flow.

Conclusion

Roller bottles are an appropriate system for short term cultures of UCB $CD34^+$ enriched cells and they allow a 16.84±2.82 fold expansion of hematopoietic progenitors from this source. The clinical efficacy of the expanded cells must be studied in detail. This data confirm the utility of RB for the expansion of hematopoietic progenitors.

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4. OVERVIEW

There are several strategies developed to expand umbilical cord blood hematopoietic stem cells *in vitro* aiming the transplantation of adult patients. We tested a roller bottle culture system to improve the nutrients and oxygen flow, which is limited in static cultures and minimize the shear stress that may be damaging the cells in bioreactor cultures. In addition, the roller bottle cultures are simple to set up and they require a relative low investment of sources. The proposed system includes the use of a culture media to perform the cultures in a CO_2-free atmosphere and low doses of cytokines.

We used the roller bottle system to culture total mononuclear cells and $CD34^+$ selected cells in batch cultures attaining HSC expansion in all the conditions tested.

For the total mononuclear cell cultures (section 3.1) we were able to expand the HSC without considerable proliferation of the total cells, which probably means that the nutrients may being used mainly for the expansion of HSC rather than for their differentiation. In the RB cultures, we achieved expansions close to our control cultures and comparable to those reported in previous publications. The Stem Pro medium, which was developed to culture HSC allowed the lower HSC expansion in RB cultures. IMDM is one of the most used media for the culture of HSC; we realized that in RB cultures this medium allows a slightly lower expansion of HSC than the L-15 medium that is first described for the culture of this type of cells. The highlights are that HSC can be expanded using CO_2-free conditions and HSC expansion is kept at a constant rate from day 5 to 13 of culture.

In the $CD34^+$ cell cultures (section 3.2) we attained even higher HSC expansions than the reported previously, also in a shorter time. In these experiments however, the total cell proliferation was higher than the HSC expansion in the tested conditions. Using IMDM, the expansion of HSC was kept for a longer period in the total mononuclear than in the $CD34^+$ cell cultures. It may be worth to test the culture of $CD34^+$ cells with the L-15 medium.

It has been reported that RB cultures are easily scalable to larger volumes; we were able to set up cultures twice the volume reported in this work with the same results. Larger volume cultures need to be performed to evaluate the top volume in which these RB provide the same result and whether this will be clinically relevant.

In vitro expanded HSC, even using bioreactors, have been proved to keep their engraftment and repopulating characteristics. These information support the hypothesis that the cells expanded in this work will be useful in transplants. However, a full testing of the cells in animal models must be conducted before using them for clinical trials.

5. CONCLUSIONS

It is possible to expand HHSC from umbilical cord blood in roller bottle cultures in different culture media supplemented with small amounts of growth factors and interleukins.

The roller bottle system is simple to operate and to scale up.

The total cell expansion achieved in the roller bottle cultures in most conditions tested is very similar to the static control cultures.

The roller bottle system with L-15 culture media allows the expansion of the hematopoietic progenitors in a CO_2-free atmosphere, at a higher extent and for larger periods than the IMDM and Stem Pro 34-SFM media in standard 5% CO_2 cultures.

The IMDM roller bottle cultures started with $CD34^+$ selected cells allow a high progenitor expansion only for a period of 5 days, whereas the same type of cultures started with total mononuclear cells, permitted the expansion for 15 days.

6. APPENDIXES

6.1 Construction and testing of the transfection vector *peGFP-N1.8*

In order to assess different protocols for non-viral transfection in HHSC, we constructed the vector peGFP-N1.8.

Methods:

Enzymatic digestions

All enzymatic restrictions of the constructs were performed using 1U of enzyme per μg of DNA. The reaction conditions were the recommended by the provider in a reaction volume of 10 μl. Digestions were incubated at 37°C for 30 min to 2 hours. Digestion products were electrophoresed in a one percent agarose gel and stained later with a solution containing 10 μg/ml Ethidium bromide. To confirm the identity of the vector, digestions were carried out with the enzymes NotI, BglII and Pst1.

DNA Isolation and Purification

Purification after plasmid isolation or enzymatic digestions was performed from a 1.5 percent low melting point agarose gel, 10μg/ml Ethidium Bromide. Digestion products, or plasmid minipreps were electrophoresed until the appropriate band was well separated from other bands. The bands were cut from the gel under UV ilumination and gel purified with QIAquick gel extraction kit (QIAgen) according to the provider's instructions.

Transformation

Competent *E-coli XL-1 blue, TOP-10 or DH5-*α cells stored at -80°C were thaw on ice for 20 min, incubated for thirty minutes with 5μl of the ligation products at 4°C, incubated for 45 seconds at 42°C and replaced on ice for 2 minutes. Two hundred and fifty micro litres of LB culture media were added and the cells were incubated 45 minutes at 37°C with continuous shaking. Cells were plated in LB, 30μg/ml Kanamycin agar plates and incubated overnight at 37°C. Transformant colonies were picked and re-grown overnight in 5 ml LB media, 30μg/ml Kanamycin.

Plasmid DNA Preparation

All constructs were obtained from overnight cultures of the kanamycin resistant colonies using the QIAprep Spin Miniprep Kit and gel purified with the QIAquick gel extraction kit (QIAgen) according to the provider's instructions. After purification, plasmids were stocked in 100ng/μl aliquots.

Mammalian Cell culture

HEK cells were maintained in IMDM supplemented with 10% FBS at 37°C in a 5 %CO_2 atmosphere.

At 70-80% confluence, culture media was eliminated; cells washed twice with PBS (Phosphate buffered saline), trypsinised with trypsin-EDTA 20mM and passaged 1:3.

Mammalian Cell Transfection

Transfection of HEK cells was performed in 24 well plates using Lipofectamine 2000 (invitrogen), according to the provider´s instructions. Briefly, the day before transfection cells were plated $5x10^4$ in 500μl of IMDM without antibiotics. For each transfection well, 0.8μg of DNA were diluted in 50 μl of serum free IMDM. In a separate tube 0.2μl of Lipofectamine 2000 were diluted in 50μl of serum free IMDM and incubated 5 minutes at RT. The content of both tubes and the mixture incubated 20 min at RT. The mixture is added to the transfection well containing the cells and free serum media. Cells are incubated at 37°C in a 5 %CO_2 atmosphere for 6 hours-8 hours, media supplemented and incubation continued for 18-72 hours prior to test transgene expression.

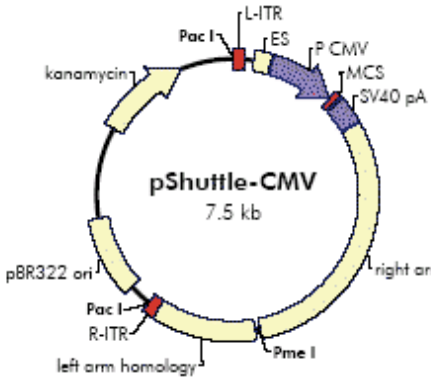
Results.

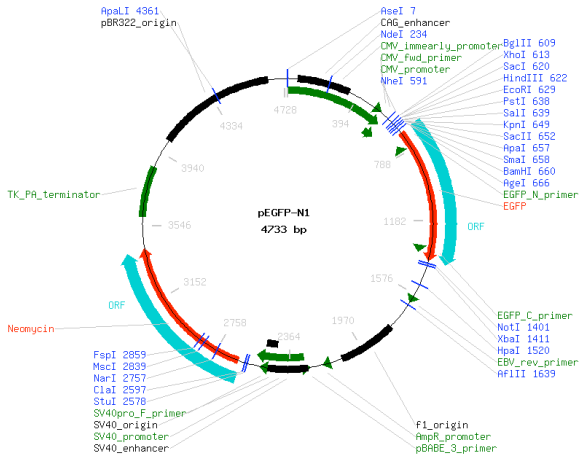
Plasmid confirmation

Figure 1, Shows the digestion maps of the vectors used to make the construct and the digestion patterns of the construct that confirm its identity.

Plasmid Testing

Figure 2 shows the fluorescence of HEK cells, 24 hours after transfection with the peGFP-N1.8 plasmid. Efficacy was between 85 to 95 %.





Mini preps

Digestión *BglII y Not*

Digestión *PstI*

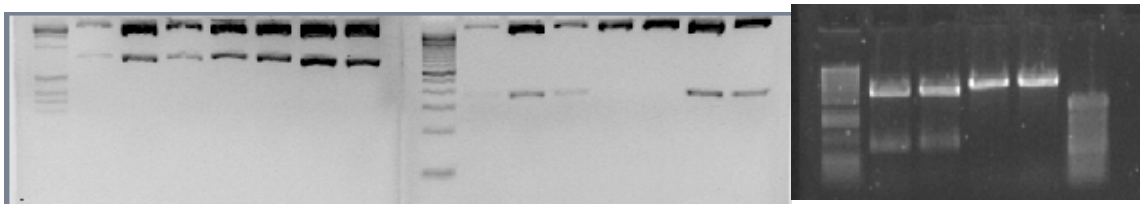


Fig 1. Construction of the vector *peGFP-N1.8*

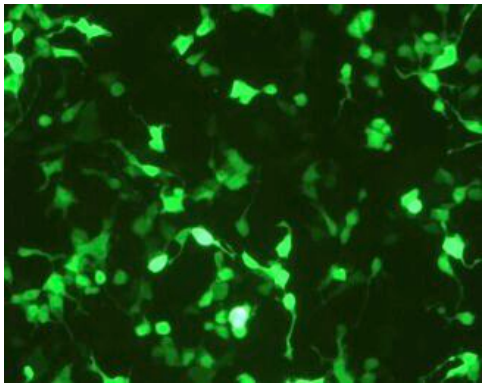


Fig 2. eGFP expressing cells after 24 hours of transfection with the plasmid peGFP-N1.8.

6.2 Non-viral transfection of hematopoietic stem cells

Retroviral gene transfer into HSC has shown several unwanted complications, such as pathogenicity, immunogenicity, uncontrollable transgene expression and in large scale it will be economically unsuitable [1]. Gene transfer into HSC is needed to research them further and to increase their clinical applications. Therefore non-viral transfection methods are a good choice, however in most cases the efficiency of the common methods in HSC is very low or the viability of the transfected cells is highly reduced.

In this work we attempted to optimize a non viral method for the transfection of cultured HSC, with low efficacy results.

Methods

Sample collection and cell separation and culture were done as described in section 3.1, total cells of different culture days were used for the transfection protocols.

Lipofection

The lipofection reagents Lipofectamine 2000 and DMRIE-C (Invitrogen) were used according to the provider´s directions for suspension cells. Several concentrations of DNA and lipidic reagent were used aiming to optimize the procedure.

Electroporation

Cells from cultures were centrifuged at 3000 rpm for 5min, culture media discarded and cells washed once with PBS and centrifuged at 3000 rpm for 5min. Cells were suspended in PBS. Cell suspension was kept on ice until electroporation. DNA was added to the cell suspension and the mixture was placed in a chilled electroporation cuvette. The Gene PulserXcell (BIO-RAD) was to 400 or 1600 μF and cells are pulsed according to manufacturer´s guidelines.

Results

Only a few attempts were positive, they are summarized in table 1. Generally, cells from a growing phase (from our cultures) showed a better result than uncultured cells. Trans gene expression efficacy ranged from < 1% to 10% approximately in the experiments, but with low level of expression in most cases.

Table 1. Transfection experiments on hematopoietic cells.

Cell Type and Concentration	Method	DNA concentration	Efficacy 24 h	Fluorescence 24h	Fluorescence 72 h
$2.35x10^5$ primary MNC cultured 3 days	Electroporation 4mm cell 217volts Pulse 57ms	30 and 60μg	1-5%	+++ transient	-
$5x10^5$ primary MNC cultured 7 days	Lipofection (Lipofectamine)	1.3μg	10%	+	+/-
$5x10^5$ primary MNC cultured 8 days	Lipofection (Lipofectamine)	1.3μg	1%	+	-
HS5 cell line	Lipofection (Lipofectamine)	0.8 y 1.6μg	2-5%	+	+

References

1. Papapetrou EP, Zoumbos NC and Athanassiadou A. (2005). Gene Therapy 12, S118–S130 Genetic modification of hematopoietic stem cells with nonviral systems: past progress and future prospects Gene Therapy 12, S118–S130