In-vitro Behavior of Human Umbilical Cord Blood Stem Cells Towards Serum Based Minimal Cytokine Growth Conditions

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Abstract We tried here to optimize the proliferation of both Hematopoietic and Mesenchymal stem cells of Umbilical Cord blood in minimal cytokine growth condition. Failing to get good results of expansion of non-adherent Hematopoietic Total Nucleated Cells and adherent Fibroblastic Mesenchymal Stem Cells derived from 10–12 ml of collected Cord blood, we designed the further experimental study by increasing the volume of Cord blood sample up to 65–70 ml. We harvested the non-adherent as well as adherent fraction separately derived from the primary culture of Umbilical Cord blood stem cells under the influence of growth promoting Cytokines or Growth Factors. The proliferation study was conducted by taking different combinations of two hematopoietic growth stimulatory Cytokines like stem cell factor (SCF) and Fms like tyrosine kinase-3Ligand (Flt3L) at concentrations (10 ng/ml, 100 ng/ml) while we preferred Mesenchymal specific growth factor i.e. basic Fibroblast growth factor (FGF- β) at its 10 ng/ml concentration for adherent cells to get optimal results. The Hematopoietic and Fibroblast Colony forming abilities of the expanded stem cells were performed through Colony Forming Unit assay. Culture Medium containing cytokine combination like SCF 100 ng/ml with Flt3L 10 ng/ml was found to be optimal for the proliferation of hematopoietic stem cells. But the number of hematopoietic colonies like Erythroid colonies generated were less in case of media supplemented with SCF & Flt3L while more number of Myeloid colonies were observed in Growth factor

supplemented media in comparison to the control one. The FGF- β supplemented media successfully enhanced the proliferation of Mesenchymal Stem Cells and exhibited its efficient Fibroblast colony forming ability. Our experimental study supports the minimal utilization of cytokines for haematopoietic and mesenchymal stem cell proliferation which may help in future safe Cord blood stem cell infusion.

Keywords Non-adherent cells · Adherent cells · Cytokines · Hematopoietic stem cells (HSCs) · Mesenchymal stem cells (MSCs)

Introduction

Human umbilical cord blood (UCB), the perinatal source of Stem Cells, is now becoming focused as an alternative to bone marrow (BM) in regenerative medicine. The mononuclear fraction of UCB is rich in stem/progenitor cells, along with their self renewal and proliferation capacity [1], they can be lineage committed for multiple differentiated cell types [2] and used for the reconstitution of the blood and immune system [3]. The CD34 antigen, an integral membrane glycoprotein, is a defining hallmark of Hematopoietic Stem Cells (HSCs) [4]. The high concentration of HSCs, i.e. CD34+/CD38–, with its rapid proliferation ability, makes the cord blood as more attractive for therapeutic application [5, 6]. The clinical results after transplantation have confirmed that either engraftment or survival significantly correlate with cell dose infused. Considering 2×10^7 /kg nucleated cells as the lower threshold for transplantation, the probability of finding UCB units suitable for recipients of 50 kg b.w. is about 40 % and this decreases to 20 % for those who weigh >70 kg.

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The reduced number of cells and progenitors available from single UCB units represents a critical issue and therefore it is necessary to establish standardized expansion procedures. Many cytokines and their combinations have been tested for proliferation of stem cells during the past 30 years. Some of these most frequently used are SCF, Flt3L, Thrombopoietin (TPO), Interleukin-3 (IL-3), and IL-6. Lam et al. (2001) [7] reported that use of SCF + Flt3L + TPO for clinical ex vivo expansion of UCB derived primitive cells. One of experimental study supported the minimal cytokine stimulation and demonstrated that TPO can functionally replace IL-3 + IL-6 + G-CSF (Granulocyte-Colony stimulating factor) for Colony Forming Unit expansion of serum free harvested UCB HSCs, when it is added to the cytokine combination of SCF + Flt3L. But TPO doesn't effectively expand Severe Combined Immuno Deficient (SCID) repopulating cells [8].

Isolation of Mesenchymal stem cells (MSCs) from UCB source is advantageous for its easy and abundant availability along with primitive nature. CD90 is one of the main markers used to identify mesenchymal stem cells in vitro [9] and enumerated both in fresh and cultured adherent UCB stem cells in our study. Although the frequency of undifferentiated MSCs is much higher in BM (0.001–0.1 %) than in UCB (0.00003 %) and some reports have even doubted the presence of MSC in UCB [10–14], UCB derived MSC can give rise up to 10^{15} cells on expansion [10, 11]. However availability of estimated clinical dose of 2×10^6 MSCs per kilogram bodyweight of adult patients has been a major challenge for transplantation. MSC can be isolated and established from only 20–63% of the cord blood units [9, 15, 16] and can undergo more population doublings than control cells, when cultured in presence of basic-Fibroblast Growth Factor (FGF- β) before they lose their proliferative capacity [17–22]. Some of the investigators compared the proliferative potential of MSCs derived from Cord Matrix, BM and UCB using 1–5 ng/ml of FGF in the growth media in one of the Indian study and observed higher fold expansion of MSCs from Cord Blood in comparison to that of BM and Cord Matrix [23]. In our study, we use 10 ng/ml FGF for the evaluation of optimal growth of MSCs from Cord blood samples.

Continued exposure of cells to GFs in-vitro is needed to prevent the cells to be affected from apoptosis. Following the harvest under the influence of GFs, the cellular products generated may undergo apoptosis if appropriate growth stimulatory factors are not present in-vivo resulting poor outcome [24]. Hence proper cytokine combination is necessitated to avoid possible exposure of patients to different cytokines with multiple in-vivo effects [25]. Most of the experimental studies have used 50–100 ng/ml or more than that concentrated SCF, as optimal for HSC expansion

[26, 27, 29–39] with other GF combinations in serum or serum free medium, however for Flt3L the working concentration for HSC expansion is mostly varied along with multiple GFs. Some of the investigators even did not include Flt3L in their hematopoietic Growth Factor Cocktail [26, 28, 30, 32, 34, 35, 37–39]. As SCF improves the homing capacity of UCB cells [40] and Flt3L leads to short term expansion of HSCs [41, 42]. In this connection, we are focusing on the establishment of optimal concentration of Flt3L s with SCF only for efficient expansion of HSCs as this is the main cytokine for the preservation of stemness of cells without further differentiation.

Our experimental study aims for the long term expansion of the HSC (non-adherent fraction) and MSCs (adherent fraction) population present in mononuclear fractions of UCB unit by supplementing minimal growth conditions using hematopoietic and mesenchymal specific growth factors in serum containing media and assess their Hematopoietic as well as Fibroblast colony forming ability.

Methodology

Collection, Processing of the Human UCB Sample

Collection of a maximum of volume 65–70 ml of residual UCB in a sterile sample collection tubes containing anticoagulant Citrate–Phosphate–Dextrose (CPD) was carried out, with the informed consent of the mothers as approved by institutional ethical committee, from the cords of the full term deliveries (gestation week: 37–40). The eligible participants were above 20 years of age with no indication of Septicemia, any other Hematological infections or complications and negative for VDRL, human immunodeficiency, hepatitis B, hepatitis C viruses. The processing time was within 7 h of collection without any sign of hemolysis or coagulation. Phosphate Buffered Saline-A or PBS-A (PBS without Ca^{2+} , Mg^{2+}), mostly used for washing of mammalian cells in the cell culture Laboratories.

The whole sample blood was diluted with PBS-A (Himedia, India) in 1:1 ratio. The stem cell population is rich with Mononuclear cells (MNCs), constituting Mononuclear Fraction (MNF) of cord blood.

UCB Mononuclear Cells (MNCs) or MNFs were recovered from the interface obtained by density gradient centrifugation (1.077 g/ml Histopaque, Sigma). Then the collected MNCs were washed twice with PBS-A and their number was evaluated by using Haemocytometer.

Primary culture of UCB MNCs

The UCB MNCs were set in culture at a plating density of $1 \times 10^6/\text{cm}^2$ in culture medium containing Dulbecco's

Modified Eagle's Medium (DMEM, PAN-BIOTECH, Germany) along with 10 % Fetal Bovine Serum (FBS, PAN-BIOTECH, Germany), and 1 % penicillin, streptomycin antibiotics (Himedia, India). Cell cultures were done at 37° C in a humidified atmosphere of 95 % air and 5 % CO₂. The medium was replaced by fresh one after 48 h. The non-adherent cells of the harvested MNCs were removed from the adherent layer and cultured separately. The culture was fed twice a week.

Isolation of Non-adherent and Adherent Fraction of Cells Under Control Condition

The floating non-adherent cells removed at each alternate day from the primary culture of Mononuclear cells representing the non-adherent fractions were set for the harvest of HSCs, while the retained adherent cells attached to the bottom constituting adherent fractions.

The non-adherent fraction of cells (HSCs) of primary culture, after removal was separately set in culture with Iscove's Modified Dulbecco's Medium with L-glutamine (IMDM, PAN-BIOTECH, Germany) containing 10 % FBS, 1 % penicillin, streptomycin antibiotics with Seeding density of $1 \times 10^4/\text{cm}^2$. On 3rd day this was continued again in alternate days. After 7th day, the media with nonadherent cultured cells were centrifuged at 1500 rpm for 5 min, then the cells were washed with PBS-A and counted in Haemocytometer.

While the retained small number of adherent fraction of primary culture was harvested in DMEM-Low Glucose (DMEM-LG, PAN-BIOTECH), 2 mmol/l L-glutamine, 10 % FBS, 1 % penicillin, and streptomycin antibiotics for 7 days, in order to increase the number of cells. On 7th day, the cells were trypsinized with using 0.25 % Trypsin/EDTA (Himedia, India) & washed twice with PBS-A for their counting.

Cell expansion and Viability were assessed by direct cell count by Trypan Blue Exclusion method using Haemocytometer.

Viability Assay

The evaluation of cell viability was conducted using 0.4 % Trypan blue (Himedia, India) by Dye exclusion method. We added 10 µl of trypan blue solution to 10 µl of the cell suspension. Afterwards, the viable cells were enumerated using an inverted microscope. The non-viable cells stained blue as they took up the dye while the viable cells remained colorless excluding the dye. For assessment of viability of cultured cells:

$$\% \text{ Viability} = \frac{\text{Number of viable cells}}{\text{Total number of viable and non-viable cells}} \times 100$$

FACS of Both Expanded Adherent and Nonadherent Fractions

A wide range of fluorophores can be used as labels in flow cytometry. Fluorophores, or simply “fluors”, are typically attached to an antibody that recognises a target feature on or in the cell; they may also be attached to a chemical entity with affinity for the cell membrane or another cellular structure [43]. We choose most commonly-used fluorescent dyes Fluorochromes like Fluorescein by its amine reactive IsothioCyanate derivative (FITC) and Phycoerythrin (PE) conjugated antibodies for FACS analysis of our study. Initially with 10–12 ml of collected UCB, two phenotypic surface markers CD 34 (HSC) and CD90 (MSC) were exclusively studied for harvested non-adherent and adherent cells respectively, under control condition. These cells from each fraction were surface stained with anti-human monoclonal antibodies i.e. FITC-conjugated CD34 and PE-conjugated CD90 (both are from BD Biosciences), after which they were kept in freeze at 4° C for 20–30 min. Washing with PBS was followed by analysis by FACS. The data generated by Flow-cytometer were analysed using CellQuest Pro Software.

Culture Condition Under Growth Promoting Cytokines

Hematopoietic GFs Supplemented Culture of Non-adherent Cells

The removed non-adherent fraction of cells (HSCs) of primary culture of UCB MNCs were studied for their expansion in presence of two of the Haematopoietic GFs like Recombinant human Stem Cell Factor (SCF, MACS, Germany), Recombinant human FMS-like tyrosine kinase-3 ligand (Flt3L, MACS, Germany) with that of the Control one. Addition of Cytokines at different combinations of concentrations i.e. (i) SCF-10 ng/ml, Flt3L-10 ng/ml (S-10 ng/ml, F-10 ng/ml), (ii) SCF-10 ng/ml, Flt3L-100 ng/ml (S-10 ng/ml, F-100 ng/ml), (iii) SCF-100 ng/ml, Flt3L-10 ng/ml (S-100 ng/ml, F-10 ng/ml) & (iv) SCF-100 ng/ml, Flt3L-100 ng/ml (S-100 ng/ml, F-100 ng/ml) to Iscove's Modified Dulbecco's Medium with L-glutamine (IMDM, PAN-BIOTECH, Germany) containing 10 % FBS, 1 % penicillin, streptomycin antibiotics, were considered for the assessment of their optimal concentration. The Seeding Density was $1 \times 10^5/\text{cm}^2$ and the culture was incubated for 10 days. Subsequent sub culturing was carried out with cell dose of 1×10^4 when culture was 70–80 % confluent. The proliferation was analyzed up to 4 weeks or 30 days. Cell Proliferation and Viability were assessed by direct cell count using Haemocytometer at each passage.

FGF- β Supplemented Adherent Mesenchymal Stem Cells (MSCs) Culture

The retained adherent cell population (MSCs) was set in culture after the complete removal of non-adherent fraction. The harvest of adherent cells with the mesenchymal growth factor recombinant human FGF- β (also known as

recombinant human basic Fibroblast Growth factor or rh FGF- β , e-Biosciences) was taken as experimental group to that of the control one (without FGF- β). Adherent cells were plated with a density of 1×10^5 cells/cm² in the medium DMEM-Low Glucose (DMEM-LG, PAN-BIO-TECH), 2 mmol/l L-glutamine, 10% FBS, 1 % penicillin, and streptomycin antibiotics along with 10 ng/ml FGF- β

Fig. 1 Primary Culture of MNCs derived from 12 ml of Cord blood. **a** Phase contrast Microscopy image of macrophages & fibroblasts in adherent fraction. **b** Culture of nonadherent cells **c** FACS analysis of

nonadherent cells. **d** Culture of adherent cells **e** FACS analysis of adherent cells

for 14 days. The passaging was done at every 7 days using 0.25 % Trypsin/EDTA (Himedia, India) and replated at a density of 4,000 cells/cm². Cultures were fed twice a week.

Both the control and experimental culture plates were subcultured at the same time for comparative analysis. Cell Proliferation and Viability were assessed by Neubauer's chamber of Haemocytometer at each passage. The Population Doubling Time (PDT) of MSCs was calculated by the formula

$$G = t \times \log 2 / \log N_t - \log N_0$$

where G is the doubling time, t is the time period between the subculturing, N_t is the no. of cells at time t and N₀ is the number of cells seeded initially i.e. t = 0.

PDT of the MSCs during their harvest at each passage was studied for both of the Control (without FGF) as well as the FGF supplemented culture plates.

Haematopoietic Colony Forming Cell Assay

Colony Forming Cell Assay was carried out with the non-adherent cells after 14 days of culture in SCF & Flt3L supplemented medium. Cell dose of approximately 2×10^5 numbers along with IMDM media with 2 % FBS for a plating concentration of 2×10^4 cells per well of 6 well plate. 400 µl of the cells were added to 4 ml of Methocult (04434, Stemcell Technologies) mixture for duplicate cultures. Then the culture plate was incubated in a water-jacketed incubator 37 °C, in 5 % CO₂, with ≥95 % humidity for 14 days. Evaluation of relative distribution of Burst Forming Unit–Erythroid (BFU-E), Colony Forming Unit–Granulocyte, Macrophage (CFU-GM) and Colony Forming Unit–Granulocyte, Erythroid, Macrophage, Megakaryocyte (CFU-GEMM) was carried out under inverted microscope as per instructions provided in the product information sheet.

In this study colonies were considered to be contained with more than 100 cells.

Colony Forming Unit-Fibroblast Assay for Adherent Cells

The expanded cells after 2nd passage of FGF supplemented Adherent cell culture, were harvested in the DMEM-LG medium having 2 mmol/l L-glutamine, 10 % FBS, 50 IU/ml penicillin, and 50 µg/ml streptomycin along with 10 ng/ml FGF-β for 14 days with the cell dose of $1-2 \times 10^5$. CFU-F count for colony containing ≥15 cells was carried out on day 14 under inverted microscope.

Statistical Analysis

The data were analyzed and graphed by using the GRAPH-PAD PRISM5 SOFTWARE. Statistical Significance was

Fig. 2 Higher frequency of fibroblast cells as observed in 65–70 ml of cord blood

determined using ANOVA and *t* test, with significant *P* value < 0.05*.

Results

Morphology & Proliferation of UCB Derived Both HSCs and MSCs

Cellular Heterogeneity in UCB MNCs Culture

The primary culture of UCB MNCs was found to be contained with three layers, when harvested in the DMEM medium containing 10 % FBS and antibiotics.

The adherent layer formed the bottom portion of culture by 8–10 days of culture of UCB MNCs. The cells of this layer contained with bigger flat cells, like fibroblasts, macrophages that attached firmly to the culture plate, considered as stromal or mesenchymal population, as shown in (Fig. 1a). The non-adherent fraction mainly constitutes two layers i.e. middle and the uppermost layer. In the middle layer, small round cells loosely attached to the lowermost adherent layer. They can be easily separated after giving small stress to the culture. The uppermost layer consisted of a large number of small rounded floating cells. The total mono nuclear cells found after processing was 1.7×10^8 derived from 10–12 ml of collected cord blood sample. We tried to expand both non-adherent as well as adherent cells separately during short term culture of stem cells but poor outcome of proliferation was observed in both the cases as observed in our previous study [44]. The confluence was not attained by non-adherent cells in culture (Fig. 1b), while only 10–12 numbers. of fibroblastic cells in adherent fraction i.e. with 3 % collected UCB units was observed (Fig. 1d).

Fig. 3 **a** Confluence attained by proliferated TNCs under the influence of SCF & Flt3L. **b** Wright Giemsa Staining. **c** Comparative analysis of expansion of TNCs between control and GFs stimulated media

Flowcytometric Analysis

The phenotypic expression of MNCs of fresh 10–12 ml of blood sample showed mean 0.713 ± 0.16 % (with S.D.) CD34+ and 0.194 ± 0.04 % (mean with S.D.) of CD90+ cells. When the expanded nonadherent cells and adherent cells under control condition was subjected to characterization with the above two markers, we got the mean value of 4.12 ± 0.39 % (with S.D.) of CD34+ cells in nonadherent fraction on 7th day of culture (Fig. 1c) while 3.08 ± 0.37 % (mean with S.D.) of CD90+ cells on 10th day of expanded adherent cells exclusively (Fig. 1e). But the number of fibroblastic elongated attached cells was found to be more in the adherent fraction expanded under control condition derived from 65–70 ml of cord blood processed within 7 h of collection (Fig. 2).

So we aimed to standardize optimal growth condition using minimal use of GFs for the proliferation of nonadherent and adherent cells from primary culture of MNCs derived from 6570 ml of collected sample.

Proliferation

Non-adherent HSCs Confluence was attended after 10 days of harvest at different combinations of concentrations of SCF & Flt3L (10 ng/ml, 100 ng/ml) indicating their efficient proliferative effect on UCB derived nonadherent population. After 10 days of incubation, nonadherent cells did not show any morphological differentiation in staining with Wright-Giemsa Stain (Fig. 3a, b).

There was sharp increase in mean (S.D.) 51.44 ± 0.32 fold expansion of TNCs in the media containing S (100 ng/ml), F (10 ng/ml) while 47.74 ± 0.21 fold increase in presence of higher concentration of both of SCF & Flt3L (100 ng/ml) in 30 days.

Increase in concentration of either GFs (S-100 ng/ml, F-10 ng/ml or S-10 ng/ml, F-100 ng/ml) or both of them (S-100 ng/ml, F-100 ng/ml) presented significant number of expanded cells than that of both GFs at their lowest concentration (S-10 ng/ml, F-10 ng/ml) with $P = 0.0021$, 0.0036 and 0.0025 respectively. While there was no

Fig. 4 Images of proliferated MSCs in FGF- β supplemented media in P1 (a) and P5 (b) passages. c Graphical presentation of cell proliferation at each passage

significant differences observed between combinations like S-100 ng/ml, F-10 ng/ml and S-10 ng/ml, F-100 ng/ml ($P = 0.2126$).

Adherent MSCs We were able to generate more number of MSCs like fibroblastic cells in control media containing DMEM-LG, 10 % FBS from 65–70 ml of collected cord blood after processing, but they never reached confluence during their harvest.

We observed 80–90 % confluence of MSCs with FGF- β (10 ng/ml) supplementation in the control media. The cells attained elongated spindle shaped fibroblastic morphology (Fig. 4a, b). In presence of FGF, cells proliferate faster reaching up to 21.52 ± 0.03 (mean with SD) fold expansion in 5th passage significantly with $P < 0.0001$ (Fig. 4c).

The shorter mean Population Doubling Time (PDT) of cells in stimulated media was found to be 37.88 ± 0.02 h in comparison to the control media significantly ($P = 0.0005$).

The Population Doubling Time (PDT) at each passage was graphically represented (Fig. 4d).

Colony Formation Assays

Haematopoietic Colony Formation Assay

The non-adherent cells responded to haematopoietic colony assay with the generation of colonies, mainly consisting of BFU-E, CFU-GM, and CFU-GEMM (Fig. 5a, b, c). Cells were plated in the density of 1×10^4 in the Methocult media & incubated for 14 days for evaluation of colonies. We represent mean (S.D.) of each type of hematopoietic colony generated in percentage (Fig. 5d). The frequency of colonies of erythroid lineage was highest (75.66 ± 2.51 %) in control media than with GF supplemented media at their highest (S-100 ng/ml, F-100 ng/ml)

Fig. 5 **a** BFU-E. **b** CFU-GM. **c** CFU-GEMM. **d** Graphical Presentation of mean number of hematopoietic colonies in percentage. **e** Fibroblast colonies in FGF supplemented media

and lowest (S-10 ng/ml, F-10 ng/ml) concentration significantly with P value <0.0001 and 0.0002 respectively.

The Cytokine combination like S-10 ng/ml, F-100 ng/ml generated comparatively higher number of myeloid based

CFU-GM colonies ($87.00 \pm 1.0\%$) than that of S-100 ng/ml, F-100 ng/ml significantly ($P = 0.0022$) while with S-100 ng/ml, F-10 ng/ml, significant differences $P < 0.0001$ was observed.

Regarding the mixed type colony formation, S-100 ng/ml, F-100 ng/ml demonstrated highest number of colonies significantly than that of Control one and S-100 ng/ml, F-10 ng/ml with $P = 0.0020$, 0.0127 respectively, but compared to S-10 ng/ml, F-100 ng/ml, the aforementioned combination didn't show any significant differences with $P = 0.0981$. Some cells with the morphological similarity to Dendrite Cells observed on the 14th day of the above assay, their number was very less (2–4 numbers.) in Control media while nearly 15–27 numbers of these cells were found in case of the media supplemented with SCF & Flt3L (data not shown).

Fibroblast Colony Forming Unit Assay

Cell dose of 1×10^5 of adherent cells were plated for the CFU-F assay with the control (DMEM-LG/10 %FBS) as well as FGF- β (10 ng/ml) supplemented media for 14 days in duplicates. We got some small and large Fibroblast colonies (Fig. 5e) through out the culture plate. Colonies with the cell number of ≥ 15 was counted for this assay. There was significant number of Fibroblast colonies in FGF supplemented media in comparison to control one ($P < 0.0001$). We repeated this experiment 10 times with the control media; control plates never generated more than 4 CFU-F colonies (Table 1).

Discussion

For the last two decades stem cell researchers have focused on the growth factors which can stimulate the proliferation of HSCs in-vitro without differentiation, generating large numbers of HSCs for clinical therapeutic application for various disease models. Felice et al. [42] demonstrated that the addition of Flt3L 50 ng/ml to SCF, interleukin-3 fusion protein (PIXY 321) or SCF, PIXY, IL-6 induced a 10 fold amplification of hematopoietic progenitors and the expansion of multipotent (CFU-GEMM) and committed (CFU-GM, BFU-E) progenitors in eight-day culture of UCB cells.

Levac et al. [8] demonstrated that in serum free culture condition, the combination of SCF (300 ng/ml) and Flt3L (300 ng/ml) is not promoting effective expansion of Cord

Blood derived progenitors. This led us to use serum based medium with minimal use of GFs like SCF and Flt3L for the proliferation of non-adherent fraction of UCB.

Our results revealed efficient expansion of HSCs with the use of minimum number of growth promoting cytokines SCF & Flt3L. In this aspect, cytokine combination SCF-100 ng/ml + Flt3L-10 ng/ml was found to be superior to other combinations of SCF + Flt3L with significance of $P < 0.0001$. Our experimental study supports the synergistic effect of SCF and Flt3L to provide a considerable proliferative stimulus to expand UCB derived non-adherent HSCs and maintain its undifferentiated state during their harvest. So, we can use SCF-100 ng/ml with 10 ng/ml of Flt3L as optimal combination in serum for expansion of HSCs in order to minimize the consumption of these costly GFs.

Regarding the haematopoietic colony formation, highest percentage of tightly packed bright red colored colonies of Erythroid lineage was observed in case of control media than GFs supplemented media. Regarding Myeloid lineage, CFU-GM was the predominant one in all combinations of concentrations of SCF & Flt3L while mixed CFU-GEMM number was very less. Cells with similar morphology to that of dendritic cells observed in the CFU assay may be due to Flt3L and GM-CSF in Methocult media [45, 46]. Still this observation needs proper identification of dendrite cells and experimentation on their role in therapeutics.

Coming to the Mesenchymal population of Cord blood, which is very very less, we are able to separate it after increasing volume of the collected blood up to 65–70 ml and decreasing the processing time i.e. within 7 h after collection. As our results reveal that none of the control culture plate of adherent MSCs attain confluence during their harvest, but at the same time 80–90 % confluence was observed after FGF- β addition to the control media with efficient generation of fibroblast colonies in FGF based CFU-F assay. One of the Indian studies reported increased 22 fold expansion of UCB MSCs upto 6th passage with PDT 44 h [23] while our study demonstrated increased fold expansion in number of fibroblastic adherent MSC like cells, found to be significant at the end of 5th passage with shorter doubling time i.e. 37.88 ± 0.02 h ($P = 0.0005$) in presence of 10 ng/ml of FGF than the control one.

Table 1 Fibroblast colony forming assay

Type of cultured cells	Mean(S.D.)* number of CFU colonies
Control adherent cells (w/o FGF)	2.5 ± 1.3
Stimulated adherent cells (with FGF)	38.5 ± 11.11

* Here the scoring value represents the mean number of Fibroblast colonies generated in the CFU-F assay for expanded adherent fraction under stimulation of 10 ng/ml FGF- β after 14 days of incubation

Conclusion

With the experimental findings cited above, our study report concludes that we can efficiently generate HSCs and MSCs from single UCB unit employing serum media on long term basis with minimal utilization of growth promoting cytokines required for a safe and effective engraftment. Further investigation on Dendrite like cells

generated in CFU assay and Proliferation of stem cells using Cord blood serum, autologous serum or serum free media containing suitable cytokine cocktail will be useful in understanding their in-vivo behavior in therapeutic application.

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Conflict of interest The authors declare that they have no competing interests.

References

- Kim SY, Park SY, Kim JM, Kim JW, Kim MY, Yang JH, et al. Differentiation of endothelial cells human umbilical cord blood AC133-CD14+ cells. *Ann Hematol*. 2005;84(7):417–22.
- Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. *Biol Blood Marrow Transplant*. 2001;7(11):581–8.
- Broxmeyer HE. Biology of cord blood cells and future prospects for enhanced clinical benefit. *Cytotherapy*. 2005;7(3):209–18.
- Sutherland DR, Keating A. The CD34 antigen: structure, biology and potential clinical applications. *J Hematother*. 1992;1:115–29.
- Bordeaux-Rego P, Luzo AC, Costa FF, Saad ST, Crosara Alberto DP. Both IL-3 and IL-6 are necessary for better ex vivo expansion of CD133+ cells from Umbilical Cord blood. *Stem Cells Dev*. 2010;19(3):413–22.
- Kadereit S, Deeds LS, Haynesworth SE, Koc ON, Kozik MM, Szekely E, et al. Expansion of LTC-ICs maintenance of p21 and BCL-2 expression in cord blood CD34+/CD38– early progenitors cultured over human MSCs as feeder layer. *Stem Cells*. 2002;20(6):573–82.
- Lam AC, Li K, Zhang XB, Li CK, Fok TF, Chang AM, et al. Preclinical ex vivo expansion of cord blood hematopoietic stem and progenitor cells: duration of culture; the media, serum supplements, and growth factors used; and engraftment in NOD/SCID mice. *Transfusion*. 2001;41(12):1567–76.
- Levac K, Karanu F, Bhatia M. Identification of growth factor conditions that reduce ex vivo cord blood progenitor expansion but do not alter human repopulating cell function in vivo. *Hematologica*. 2005;90:166–72.
- Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood or adipose tissue. *Stem cells*. 2006;24(5):1294–301.
- Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult Bone marrow is a rich source of human mesenchymal ‘stem’ cells but umbilical cord and mobilized adult blood are not. *Br J Haematol*. 2003;121(2):368–74.
- Pozzi S, Lisini D, Podestà M, Bernardo ME, Sessarego N, Piaggio G, et al. Donor multipotent mesenchymal stem cells may engraft in paediatric patients given either cord blood or bone marrow transplantation. *Exp Haematol*. 2006;34(7):934–42.
- Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood*. 2004;103(5):1669–75.
- Pedikogianni C, Dimitriou H, Stiakaki E, Martimianaki G, Kalmanti M. Could cord blood be a source of mesenchymal stromal cells for clinical use? *Cytotherapy*. 2008;10(5):452–9.
- Solves P, Mirabet V, Planelles D, Carbonell-Uberos F, Roig R. Influence of volume reduction and cryopreservation methodologies on quality of thawed umbilical cord blood units for transplantation. *Cryobiology*. 2008;56(2):152–8.
- Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells*. 2004;22(4):625–34.
- Rebelatto CK, Aguiar AM, Moretão MP, Senegaglia AC, Hansen P, Barchiki F, et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Exp Biol Med* (Maywood). 2008;233(7):901–13.
- Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology*. 1997;138(10):4456.
- Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp Haematol*. 2000;28(6):707–15.
- Mastrogiacomo M, Cancedda R, Quarto R. Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. *Osteoarthritis Cartil*. 2001;9(Suppl A):S36.
- Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, et al. Ex vivo enrichment of mesenchymal cell progenitors fibroblast growth factor-2. *Exp Cell Res*. 2003;287(1):98–105.
- Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun*. 2001;288(2):413–9.
- Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone marrow derived mesenchymal stem cells. *Tissue Eng Part A*. 2010;16(3):1009–19.
- Shetty P, Cooper K, Viswanathan C. Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. *Asian J Transfus Sci*. 2010;4(1):14–24.
- McNiece I. Delivering cellular therapies: lessons learned from ex vivo culture and clinical applications of hematopoietic cells. *Semin Cell Dev Biol*. 2007;18(6):839–45.
- Vial T, Descotes J. Clinical toxicity of cytokines used as hematopoietic growth factors. *Drug Saf*. 1995;13:371–406.
- Briddell RA, Kern BP, Zilm KL, Stoney GB, McNiece IK. Purification of CD34+ cells is essential for optimal ex vivo expansion of umbilical cord blood cells. *J Hematother*. 1997;6(2):145–50.
- Dao MA, Creer MH, Nolte JA, Verfaillie CM. Biology of umbilical cord blood progenitors in bone marrow niches. *Blood*. 2007;110(1):74–81.
- Chivu M, Diaconu CC, Bleotu C, Alexiu I, Brasoveanu L, Cernescu C. The comparison of different protocols for expansion of umbilical-cord blood hematopoietic stem cells. *J Cell Mol Med*. 2004;8(2):223–31.
- da Silva CL, Goncalves R, Crapnell KB, Cabral JM, Zanjani ED, Almeida-Porada G. A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. *Exp Hematol*. 2005;33(7):828–35.
- Guenechea G, Segovia JC, Albella B, Lamana M, Ramirez M, Regidor C, et al. Delayed engraftment of nonobese diabetic/severe combined immunodeficient mice transplanted with ex vivo-expanded human CD34(+) cord blood cells. *Blood*. 1999;93(3):1097–105.
- Jang YK, Jung DH, Jung MH, Kim DH, Yoo KH, Sung KW, et al. Mesenchymal stem cells feeder layer from human umbilical cord blood for ex vivo expanded growth and proliferation of hematopoietic progenitor cells. *Ann Hematol*. 2006;85(4):212–25.

32. Jiang XS, Chai C, Zhang Y, Zhuo RX, Mao HQ, Leong KW. Surface-immobilization of adhesion peptides on substrate for *ex vivo* expansion of cryopreserved umbilical cord blood CD34+ cells. *Biomaterials*. 2006;27(13):2723–32.
33. Martin-Rendon E, Hale SJ, Ryan D, Baban D, Forde SP, Roubelakis M, et al. Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia. *Stem Cells*. 2007;25(4):1003–12.
34. McNiece IK, Almeida-Porada G, Shpall EJ, Zanjani E. *Ex vivo* expanded cord blood cells provide rapid engraftment in fetal sheep but lack long-term engraftment potential. *Exp Hematol*. 2002;30(6):612–6.
35. McNiece I, Kubegov D, Kerzic P, Shpall EJ, Gross S. Increased expansion and differentiation of cord blood products using a two-step expansion culture. *Exp Hematol*. 2000;28(10):1181–6.
36. Oswald J, Steudel C, Salchert K, Joergensen B, Thiede C, Ehn-inger G, et al. Gene-expression profiling of CD34 hematopoietic cells expanded in a collagen I matrix. *Stem Cells*. 2006;24(3):494–500.
37. Robinson SN, Ng J, Niu T, Yang H, McMannis JD, Karandish S, et al. Superior *ex vivo* cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. *Bone Marrow Transp*. 2006;37(4):359–66.
38. Shpall EJ, Quinones R, Giller R, Zeng C, Baron AE, Jones RB, et al. Transplantation of *ex vivo* expanded cord blood. *Biol Blood Marrow Transp*. 2002;8(7):368–76.
39. Wang JF, Wang LJ, Wu YF, Xiang Y, Xie CG, Jia BB, et al. Mesenchymal stem/progenitor cells in human umbilical cord blood as support for *ex vivo* expansion of CD34(+) hematopoietic stem cells and for chondrogenic differentiation. *Hematologica*. 2004;89(7):837–44.
40. Zheng Y, Sun A, Han ZC. Stem cell factor improves SCID-repopulating activity of human umbilical cord blood-derived hematopoietic stem/progenitor cells in xenotransplanted NOD/SCID mouse model. *Bone Marrow Transp*. 2005;35:137–42.
41. De Felice L, Di Pucchio T, Breccia M, Agostini F, Mascolo MG, Guglielmi C, et al. Flt3L enhances the early stem cell compartment after *ex vivo* amplification of umbilical cord blood CD34+ cells. *Bone Marrow Transp*. 1998;22(Suppl 1):S66–7.
42. De Felice L, Di Pucchio T, Grazia Mascolo M, Agostini F, Breccia M, Guglielmi C, et al. Flt3L induces the *ex-vivo* amplification of umbilical cord blood committed progenitors and early stem cells in short-term cultures. *British J Haematol*. 1999;106:133–41.
43. Loken MR. Immunofluorescence techniques in flow cytometry and sorting. 2nd ed ed. New York: Wiley; 1990. p. 341–53.
44. Mantri S, Mohapatra PC. In-vitro study of proliferation in short term cultured Umbilical Cord blood stem cells. *Orissa Med J*. 2013;33(1):108–11.
45. Canque B, Camus S, Dalloul A, Kahn E, Yagello M, Dezutter-Dambuyant C, et al. Characterization of dendritic cell differentiation pathways from cord blood CD34⁺CD7⁺CD45RA⁺ hematopoietic progenitor cells. *Blood*. 2000;96:3748–56.
46. McKenna HJ, Stocking KL, Miller RE, Brasel K, Smedt TD, Maraskovsky E, et al. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 2000;95:3489–97.