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Ex-vivo Expansion of Cord Blood Hematopoietic Stem Cells in Serum-Free Medium with Feeder Layer - A New Hope for Successful Cord Blood Transplantation in Adults

Asmaa M. Borg, Fatma Auf, Farha El-Chennawi, Zakaria.F.Lotfy

Clinical Immunology Unit, Department of Clinical Pathology, Mansoura Research Centre for Cord Stem Cells (MARC-CSC), Faculty of Medicine, Mansoura University, Mansoura, Egypt

Corresponding Author: Asmaa M. Borg, Clinical Immunology Unit, Department of Clinical Pathology, Mansoura Research Centre for Cord Stem Cells (MARC-CSC), Faculty of Medicine, Mansoura University, Mansoura, Egypt
E-mail: dr_asmaaborg@mans.edu.eg

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ABSTRACT

Background: Umbilical cord blood is widely regarded as a viable option for allogeneic hematopoietic stem cell transplantation (HSCT) and serves as a potential alternative to bone marrow due to its numerous advantages. These include a non-invasive collection process, a high concentration of hematopoietic stem and progenitor cells, and a lower risk of graft rejection. However, its application in adult patients is limited by the suboptimal dose of stem cells available in a single umbilical cord blood unit. This insufficient cell dose increases the risk of engraftment failure and post-transplant mortality, posing a significant challenge for its use in adult populations.
Materials and Methods: This study aims to develop a protocol for expanding umbilical cord blood mononuclear cells (UCB-MNCs) using a serum-free culture medium called StemSpan, supplemented with a mesenchymal stem cell (MSC) feeder layer and a combination of growth factors. The growth factors used include stem cell factor (SCF), thrombopoietin (TPO), fibroblast growth factor-1 (FGF-1), and heparin. The expansion culture was applied to 20 UCB samples and maintained over a period of 13 days. Data collected from the experiments were analyzed using the SPSS program (Statistical Package for the Social Sciences) for Windows, version 21.
Results: The protocol led to a notable increase in the viable mononuclear cell counts, the absolute hematopoietic stem and progenitor cell counts, and the clonogenic progenitors.
Conclusion: This designed protocol could support the expansion of the umbilical cord blood mononuclear cells, including hematopoietic stem and progenitor cells, which could provide hope for better engraftment in adult patient transplantations. The designed protocol could effectively promote the expansion of umbilical cord blood mononuclear cells, particularly hematopoietic stem and progenitor cells. This advancement offers promising potential for improving engraftment outcomes in adult patient transplantations.

Keywords: Hematopoietic stem cells; Progenitor cells; Mesenchymal stem cells; Ex-vivo expansion

INTRODUCTION

The umbilical cord blood (UCB) provides an alternative approach to allogeneic bone marrow transplantation in the management of numerous life-threatening diseases[1]. It has become one of the most important sources of stem cells for transplantation because it has several advantages over the use of bone marrow or mobilized peripheral blood stem cells, including quick availability, a non-invasive collection process, the fruitfulness of hematopoietic stem and progenitor content, and greater immune tolerance[2]. Umbilical cord stem cells were used more in the treatment of children than adults, as the amount of cells extracted from the umbilical cord blood of one newborn is not sufficient to supply stem and progenitor cells required to treat an adult patient, which may increase the possibility of engraftment failure and death[3].

Many studies tested various approaches to overcome these limitations and improve the engraftment of cord blood, such as haploidentical hematopoietic stem cells (HSCs) co-transplantation[4], enhancement of transplant homing[5], intraosseous infusion[6], dual cord blood transplantation[7], and in-vitro expansion of HSCs. That appears to be the most straightforward technique to provide clinically valid transplants from a limited starting dose [8].

The designation of the optimal culture conditions is the major focus of HSC expansion protocols. The expansion protocol must be as close as possible to in vivo hematopoiesis while keeping the stemness of the HSCs [9, 10.]

Human mesenchymal stem cells (MSCs) have been successfully applied as scaffolds for the expansion and support of HSCs. MSCs act through cell-to-cell direct contact. Apart from secreting a diversity of cytokines, including SCF, TPO, and stromal cell-derived factor 1 (SDF-1), MSCs are present in the bone marrow and act as a natural scaffold through which the HSCs differentiate and proliferate[11,12]. Huge quantities of MSCs with great proliferative ability and low cell doubling times have been isolated in previous protocols from the umbilical cord of Wharton's jelly, and the stemness of these cells continued longer than bone marrow-mesenchymal stem cells in vitro[13].

Scholars have proposed different protocols for HSC expansion from UCB using various combinations of culture media, cytokines, and growth factors [14]. Most of them unite on the use of three early recombinant hematopoietic growth factors: stem cell factor (SCF), thrombopoietin (TPO), and Flt-3-ligand or fibroblast growth factor-1 (FGF-1), recognized to be essential for the growth, maintenance of stemness and pluripotency, and preventing cell apoptosis [15].

Most existing protocols for expanding hematopoietic stem cells (HSCs) rely heavily on large quantities of growth factors, which are prohibitively expensive and pose a significant financial barrier for our work. To address this challenge, we explored cost-effective alternatives and developed the concept of a nutrient layer. This scaffold-like system serves two critical purposes: (1) it reduces dependence on exogenous growth factors by providing a supportive microenvironment, and (2) it acts as a physical scaffold to enhance cell adhesion and proliferation. By integrating this dual-function approach, we aim to minimize costs while maintaining or improving HSC expansion efficiency.

MATERIALS AND METHODS

This in-vitro experimental study was conducted at the Mansoura Research Centre for Cord Stem Cells (MARC-CSC) between 2018 and 2019. Umbilical cord tissue samples (*n* = 20) were collected consecutively from full-term, uncomplicated pregnancies involving healthy women aged 20–35 years. All participants underwent elective cesarean sections at the Department of Obstetrics and Gynecology, Mansoura University Hospitals. Specimens were selected based on predefined inclusion and exclusion criteria to ensure consistency across the study cohort.

Inclusion Criteria: a) Umbilical cord blood collection must not interfere with the infant's delivery process.

b) Collections must be performed by trained personnel certified in sterile cord blood handling, including screening, processing, transportation, and storage protocols.

c) Procedures must adhere to strict aseptic conditions to prevent contamination.

Exclusion Criteria: a) Risk of contamination (Samples collected from vaginal deliveries due to higher contamination risk in non-sterile environments). Only specimens obtained via cesarean section under controlled aseptic conditions were included. b) Delayed processing (Specimens not transported to the processing facility within the predefined timeframe e.g., >24 hours post-collection). C) Maternal or gestational complications: Pathological pregnancies, including

Gestational diabetes, Preeclampsia, Intrauterine infection, Placenta previa. Written informed consent was obtained from all participating mothers. The study protocol was approved by the Institutional Research Board (IRB) at Mansoura Faculty of Medicine (Approval Number: MD.16.04.38).

1. Umbilical cord blood Collection: Umbilical cord blood was collected using the *in-utero* technique, as described by Bassiouny et al[16].

2. Isolation of MSCs from Wharton's jelly and their culture: This step was done using the explant method, the same technique as reviewed in Goyal et al[17]. Preparation of culture medium: Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM) (HyCloneTMonzaTM Basel, Switzerland) was supplemented with 15% FBS (Sigma-AldrichTM USA) + 0.5% 2 mM L Glutamine (Sigma-Aldrich™- USA) and 0.5% penicillin/streptomycin solution (100 U/mL) (LonzaTM, Basel, Switzerland). This was mixed well and warmed in a 37 °C water path.

Cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2, and the medium was replaced every 3 days. Upon reaching 70–80% confluence, cells were detached using 0.25% trypsin-EDTA and subcultured. This passaging protocol was repeated until Passage 7, after which cells were harvested by trypsinization, cryopreserved in liquid nitrogen (-180°C), and stored for downstream applications[17].

3. MSCs characterization: According to the International Society for Cellular Therapy (ISCT) guidelines, mesenchymal stem/stromal cells (MSCs) must meet three criteria for identification: (1) plastic adherence in culture, (2) fibroblast-like morphology, and (3) ≥95% expression of MSC surface markers (e.g., CD90, CD105, CD73) combined with ≤2% expression of hematopoietic lineage markers (CD45, CD34)[18]. While the MSCs isolated in this study adhered to these minimal ISCT standards for surface marker expression and morphological characteristics, they were not functionally validated through in vitro differentiation into adipocytes, osteoblasts, or chondrocytes, as recommended by the guidelines.

4. UCB-MNCs Isolation: Low-density umbilical cord blood mononuclear cells (UCB-MNCs) were isolated using Ficoll-Paque™ density gradient centrifugation (Lymphosep, Biowest™, Nuaille, France). Buffy coats were collected into sterile 15 mL Falcon tubes, resuspended in an equal volume of RPMI 1640 medium (Biosera™, Nuaille, France), and centrifuged at 2000 rpm for 10 minutes to remove residual platelets and debris. Cells were then washed, and an aliquot was taken for viability assessment and counting. To ensure adequate cell yields for all 20 cultures, MNCs from three independent cord blood units were pooled and allocated as follows: **Unit 1:** Cultures 1–4, **Unit 2:** Cultures 5–10, and **Unit 3:** Cultures 11–20. Cryopreserved cells were thawed and expanded as needed for subsequent experiments.

5. Preparing the Wharton's jelly- MSCs feeder layer: Wharton's jelly-derived mesenchymal stromal cell (WJ-MSC) feeder layers were established as follows:

1. Cell Seeding: MSCs (Passage 4–6) were seeded at a density of 3,000 cells/cm² into T-25 flasks pre-coated with a 0.1% gelatin solution (Sigma-Aldrich™, USA) and cultured under standard conditions (37°C, 5% CO_2) until 70–80% confluence [19].
2. Mitotic Inactivation: At subconfluence, cells were mitotically inactivated by replacing the medium with DMEM supplemented with 10% FBS and 0.5 µg/mL Mitomycin C (Stem Cell Technologies™, Canada; Cat.# 73272) for 2.5 hours.
3. Post-Treatment Processing: After incubation, cells were washed twice with PBS and maintained in fresh, pre-warmed DMEM for 24 hours prior to co-culture initiation [19, 20].

6. Culture of UCB-MNCs UCB- MNCs

A total of 50,000 viable cells were plated onto a mitomycin C-treated mesenchymal stromal cell (MSC) feeder layer using StemSpan™ SFEM serum-free expansion medium (Stem Cell Technologies, Canada; Cat# 09600). The culture medium was supplemented with the following components:

- 10 ng/mL stem cell factor (SCF) (R&D Systems, Minneapolis, USA; Cat# 255-SC),
- 20 ng/mL thrombopoietin (TPO) (Stem Cell Technologies; Cat# 02522),
- 10 ng/mL fibroblast growth factor-1 (FGF-1) (Stem Cell Technologies; Cat# 78003),
- 10 µg/mL heparin solution (Stem Cell Technologies; Cat# 07980).

Cultures were maintained at 37°C in a 5% CO_2 incubator for 13 days. On days 7 and 10 of the culture period, the medium was replenished with fresh, pre-warmed StemSpan™ SFEM containing the same growth factors at identical concentrations.

7. Cultured MNCs assessment: Cell counts and viability assessments were performed on days 3, 7, 10, and 13 of the culture period using an Olympus R1 automatic cell counter (Tokyo, Japan). On day 13, after completing the final cell count and viability

assessment, the cultured cells were harvested from the flasks for further analysis, including flow cytometry and clonogenic assays. A fold-increase in viable mononuclear cell (MNC) counts was calculated to evaluate expansion during the culture period.

Uncultured (freshly isolated) mononuclear cells (MNCs) and their expanded counterparts post-culture were analyzed by flow cytometry using a panel of antibodies specific for hematopoietic stem and progenitor cells (HSPCs).

Flow cytometry analysis was performed using a BD Accuri™ C6 Plus flow cytometer (Becton Dickinson). Stem-Kit™ reagents (Beckman Coulter) containing monoclonal antibodies targeting CD34 and CD45 were used to identify hematopoietic stem and progenitor cells (HSPCs). Viability was assessed with 7-aminoactinomycin D (7-AAD) to exclude dead cells. Viable cells were identified by initial gating on a histogram based on 7-AAD negativity. A sequential gating strategy was applied to identify hematopoietic stem and progenitor cells (HSPCs):

1. **CD45+ selection**: From the initial viable cell population (7-AAD-negative), cells were first gated on CD45+ to isolate hematopoietic lineage cells.
2. **CD34+/CD45+ co-expression**: From the CD45+ population, cells co-expressing CD34 and CD45 were selected.
3. **CD45+ dim refinement**: Finally, a subset of CD34+ cells with low CD45 expression (CD45+ dim) was gated, defining the HSPC population (CD34+/CD45+ dim).

Mononuclear cells (MNCs) from three uncultured cord blood (CB) samples and post-expansion cells from 20 cultured samples were plated in semi-solid methyl cellulose medium (Methocult™ H4035, Stem Cell Technologies), optimized for myeloid colony formation (without erythropoietin). The medium contained recombinant cytokines (IL-3, GM-CSF, G-CSF, and SCF) to support the growth of colony-forming units (CFUs), defined as progenitor cells capable of forming morphologically distinct colonies (CFU-M, CFU-G, and CFU-GM).

After 14 days of incubation at 37°C, colonies were counted and classified based on morphology. Cells were plated at 15,000 cells/mL, with 1.1 mL per well (16,500 MNCs/well). To normalize CFU frequency to 10^5 MNCs, colony counts were multiplied by 6.06 (derived from 100,000 ÷ 16,500). Fold changes in CFU counts were calculated by comparing post-expansion CFU values to baseline (preculture) levels.

Statistical analysis

Data were analyzed using the SPSS program for Windows (version 21). The normality of data was first tested with a one-sample Kolmogorov-Smirnov test. Continuous variables were offered as median (min-max) for non-parametric data. The following statistical tests were used for comparison: Wilcoxon signed rank test: two paired groups (nonparametric) and Friedman test: More than 2 paired groups (nonparametric).

RESULT

We successfully isolated millions of mesenchymal stromal cells (MSCs) from Wharton's jelly and expanded them *in vitro* through passage 7 without loss of proliferative capacity (Figure 1). Flow cytometry confirmed the MSCs' immunophenotype, demonstrating strong positivity for the MSC-associated markers CD90, CD105, and CD73 (>95% expression) and absence of the hematopoietic marker CD45 (<2% expression), consistent with ISCT criteria (Figure 2). To support hematopoietic stem and progenitor cell (HSPC) expansion, we established a feeder layer system using Wharton's jelly-derived MSCs. Mononuclear cells (MNCs) isolated from cord blood were first analyzed via flow cytometry to assess baseline surface marker profiles (Figure 3). The mononuclear cells (MNCs) were co-cultured with MSC feeder layers under optimized ex vivo conditions. Following expansion, the hematopoietic cells were re-evaluated by flow cytometry to confirm preservation of HSPC markers (Figure 4).

Viable MNC counts at different follow-up periods (0, 3rd, 7th, 10th, and 13th days of the culture) and viability assessment by Trypan blue are detailed in (Table 1) and (Table 2).

The flow cytometric analysis of the preculture MNCs (Figure 3) and the expanded MNCs (Figure 4) was used to calculate the percentage of dim double-positive (CD34+/CD45+) hematopoietic stem and progenitor cells. The percentage then was multiplied by the viable MNCs absolute count to obtain the

absolute stem and progenitor cell count for each specimen (table 3). Stem and progenitor cell percentages improved significantly (p value <0.001) in the post-culture samples, and the median fold change in the percentage was 3.8 (1.53–0.92) (Table 3). The CFU colony count showed a statistically significant increase in the expanded samples in comparison with the preculture samples (Figure 5 and Table 4.

Table 1: Viable MNCs count assessment and viability assessment by Trypan blue

Sample number	Day 0	Day 3	Day 7	Day 10	Day 13
	Number of viable selected MNCs seeded	Viable count (% viability)	Viable count (% viability)	Viable count (% viability)	Viable count (% viability)
1	50,000	40,600 (28%)	22,440 (20 %)	89,760 (33 %)	1,600,000 (72 %)
2	50,000	48,865 (29 %)	37,455 (23 %)	432,300 (52 %)	1,050,000 (66 %)
3	50,000	396,900 (63 %)	306,900 (65 %)	254,430 (68 %)	905,000 (62 %)
4	50,000	63,440 (52 %)	37,455 (45 %)	584,100 (70 %)	1,200,000 (69 %)
5	50,000	854,700 (61 %)	3,668,500 (64 %)	8,400,000 (82 %)	8,750,000 (70 %)
6	50,000	158,550 (67 %)	1,023,000 (77 %)	1,395,000 (75 %)	3,950,000 (60 %)
7	50,000	4,935,000 (82 %)	4,488,000 (80 %)	3,232,500 (74 %)	3,320,000 (58 %)
8	50,000	1,414,000 (70 %)	1,298,000 (61 %)	5,407,500 (48 %)	12,000,000 (83 %)
9	50,000	285,600 (51 %)	1,848,000 (59 %)	3,570,000 (70 %)	7,350,000 (47 %)
10	50,000	254,100 (76 %)	6,765,000 (82 %)	3,840,000 (43 %)	15,400,000 (84 %)
11	50,000	2,681,000 (79 %)	671,000 (73 %)	6,090,000 (77 %)	8,390,000 (69 %)
12	50,000	889,000 (50 %)	2,843,500 (70 %)	9,825,000 (56 %)	19,300,000 (80 %)
13	50,000	1,015,000 (27 %)	2,117,500 (77 %)	4,417,500 (40 %)	6,940,000 (55 %)
14	50,000	1,792,000 (82 %)	8,690,000 (77 %)	12,750,000 (87 %)	12,000,000 (49 %)
15	50,000	222,250 (78 %)	1,149,500 (61 %)	1,635,000 (72 %)	6,890,000 (75 %)
16	50,000	1,809,500 (79 %)	8,085,000 (83 %)	3,232,500 (82 %)	3,720,000 (59 %)
17	50,000	602,000 (51 %)	1,721,500 (28 %)	2,250,000 (35 %)	4,400,000 (36 %)
18	50,000	6,650,000 (82 %)	2,046,000 (70 %)	12,975,000 (62 %)	7,300,000 (68 %)
19	50,000	714,000 (68 %)	9,185,000 (85 %)	9,150,000 (86 %)	6,350,000 (72 %)
20	50,000	714,000 (65 %)	448,800 (72 %)	9,450,000 (85 %)	6,210,000 (42 %)

Table 2: Statistical analysis of viable MNCs counts at different follow up periods

Viable MNCs counts	Day 0	Day 3	Day 7	Day 10	Day 13	Friedman's Q value	p-value of Friedman's test
Median (Min-Max)	50000 (50000-50000)	714,000 (40600-6650000)	1,784,800 (22440-9180000)	3,705,000 (89760-13000000)	6,620,000 (905000-19300000)	50.12	<0.001*
P Vs. 0d $	-	<0.001*	<0.001*	<0.001*	<0.001*		
P Vs. 3d $	-	-	0.079	<0.001*	<0.001*		
P Vs. 7d $	-	-	-	0.015*	0.002*		
P Vs. 10d $	-	-	-	-	0.044*		

Significant p <0.05, $: Wilcoxon signed rank test was used.
P Vs. 0 d $: There was significant increase in the viable MNCs count (p value<0.001) at day 3,7,10 & 13 in comparison with day 0 viable MNCs count.
P Vs. 3d $: There was significant increase in the viable MNCs count (p value<0.001) at day 10 & 13 in comparison with day 3 viable MNCs count, but no significant increase in viable MNCs count in day 7 in comparison with day 3 (p > 0.05).
P Vs. 7d $: There was significant increase in the viable MNCs count (p value<0.001) at day 10 & 13 in comparison with day 7 viable MNCs count.
P Vs. 10d $: There was significant increase in the viable MNCs count (p value<0.001) at day13 in comparison with day 10 viable MNCs count.

Table 3: Flow cytometry assessment of stem and progenitor cells

Sample	Absolute stem & progenitor cell count (percentage %)		Fold change in absolute stem & progenitor cell counts
	Day 0 (count/50000 viable cultured cells)	Day 13 (count/total viable cells harvested at the end of the culture period)	
1.	194 (1%)	120,495 (7.53%)	620
2.	194 (1%)	42,958 (4.09%)	221
3.	194 (1%)	35,808 (4%)	184.5
4.	194 (1%)	111,374 (4.83%)	573
5.	1079 (2.16%)	330,328 (3.7%)	306
6.	1079 (2.16%)	129,567 (3.3%)	120
7.	1079 (2.16%)	166,148 (5.2%)	154
8.	1079 (2.16%)	794,542 (6.6%)	736
9.	1079 (2.16%)	340,149 (4,6%)	315
10.	1079 (2.16%)	615,947 (4%)	571
11.	1235 (2.46%)	742,607 (8.9%)	601
12.	1235 (2.46%)	1,613,441 (8.4%)	1306
13.	1235 (2.46%)	845,969 (12.2%)	685
14.	1235 (2.46%)	1,042,397 (8.7%)	844
15.	1235 (2.46%)	1,391,930 (20.2%)	1127
16.	1235 (2.46%)	260,360 (7%)	211
17.	1235 (2.46%)	1,072,539 (24.4%)	868
18.	1235 (2.46%)	1,374,299 (18.8%)	1113
19.	1235 (2.46%)	621,319 (9.8%)	503
20.	1235 (2.46%)	615,209 (9.9%)	498

Stem cell percentage (%)	Pre	Post	Fold change	Wilcoxon signed rank test	p
Median (Min-Max)	2.31 (1.00-2.46)	7.96 (3.30-24.4)*	3.80 (1.53-9.92)	3.92	<0.001*

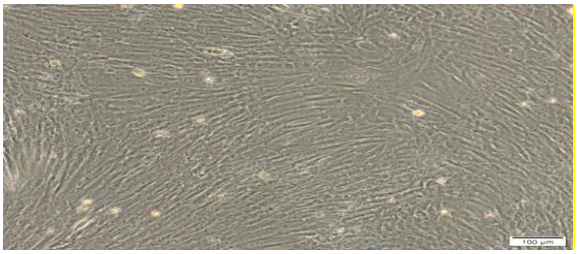
Assessment of specimens was done before culture (day 0) and at the end of culture period (day 13): The day 0 absolute count was calculated by multiplying the flow cytometric ratio of dim double positive (CD34 & CD45) cells by the number of viable events at this setting then the resulted counts was corrected to obtain the absolute counts of stem cells/ 50000 viable cells cultured at day 0, the day 13 absolute counts was calculated by multiplying the flow cytometric ratio of dim double positive (CD34 & CD45) cells by the number of viable events at this setting then the resulted counts was corrected to obtain the absolute counts of stem cells/ counted viable cells at day 13 for each sample. The percentage of dim CD34+/ CD45+ dim cells (Stem & progenitor cells) was estimated by flow cytometry for all 20 samples, the percentage was multiplied by the viable MNC count of the specimen at this day to obtain the absolute stem & progenitor cell count for each specimen. Fold change in absolute stem & progenitor cell counts was calculated by dividing day 13 by day 0 absolute counts for each specimen. **Wilcoxon signed rank test used:** *the median percentage improved significantly (p value <0.001) at day 13.

Table 4: Colony forming unit (CFU) assay (clonogenic assay): Average CFU count / 10^5 MNCs

Sample	CFU count		Fold change
	Day 0	Day 13	
1.	576	667	1.16
2.	576	1515	2.63
3.	576	1054	1.83
4.	576	1757	3.05
5.	515	7272	14.12
6.	515	6666	12.94
7.	515	1394	2.71
8.	515	2060	4.00
9.	515	1212	2.35
10.	515	4242	8.24
11.	1364	3636	2.67
12.	1364	4848	3.55
13.	1364	3030	2.22
14.	1364	7272	5.33
15.	1364	4242	3.11
16.	1364	3333	2.44
17.	1364	4545	3.33
18.	1364	4696	3.44
19.	1364	2181	1.60
20.	1364	6060	4.44
Median (Min-Max)	970 (515-1364)	3484.5 (667-7272)	3.08 (1.16-14.12)

Average CFU count / 10^5 MNCs assessed preculture (day 0) & post culture (day 13) & calculation of fold change in the CFU counts by dividing day 13 on day o counts. It is important to mention that only three Methocult cultures were done at day 0, one Methocult culture for each CB bag used to separate the MNCs, that explain why the number of colonies at day 0 was the same for samples (1-4), (5-10) & (11-20).

Wilcoxon signed rank test used and it shows statistically significant increase in the post culture CFU counts (p-value<0.001) in comparison with the preculture CFU counts.



(a)

Cell Count Report

• File name
p3 to p4

• Date
26 Jan., 2019 14:08

• Cell count results
Total cell concentration: 1.12 x 10^6 cells/mL
Live cell concentration: 8.93 x 10^5 cells/mL
Dead cell concentration: 2.27 x 10^5 cells/mL
Viability: 79.8 %
Average cell size: 31.4 µm
Total cell number: 247
Live cell number: 197
Dead cell number: 50

• Protocol
Protocol name: NEW
Dilution factor: 2
Min. cell size: 3 µm
Max. cell size: 60 µm
Size gating: 3 ~ 60 µm
Noise reduction: 5
Roundness: 60 %
Declustering level: Medium

(b)

Cell Count Report

• File name
p5 to p6

• Date
26 Jan., 2019 14:41

• Cell count results
Total cell concentration: 5.22 x 10^6 cells/mL
Live cell concentration: 4.55 x 10^6 cells/mL
Dead cell concentration: 6.71 x 10^5 cells/mL
Viability: 87.2 %
Average cell size: 13.7 µm
Total cell number: 1152
Live cell number: 1004
Dead cell number: 148

• Protocol
Protocol name: NEW
Dilution factor: 2
Min. cell size: 3 µm
Max. cell size: 60 µm
Size gating: 3 ~ 60 µm
Noise reduction: 5
Roundness: 60 %
Declustering level: Medium

(C)

Cell Count Report

• File name
p5 to p6

• Date
26 Jan., 2019 14:41

• Cell count results
Total cell concentration: 5.22 x 10^6 cells/mL
Live cell concentration: 4.55 x 10^6 cells/mL
Dead cell concentration: 6.71 x 10^5 cells/mL
Viability: 87.2 %
Average cell size: 13.7 µm
Total cell number: 1152
Live cell number: 1004
Dead cell number: 148

• Protocol
Protocol name: NEW
Dilution factor: 2
Min. cell size: 3 µm
Max. cell size: 60 µm
Size gating: 3 ~ 60 µm
Noise reduction: 5
Roundness: 60 %
Declustering level: Medium

(D)

Figure 1: (a): Mesenchymal stem cells in culture (Passage 3) under the inverted microscope (40×).
(b-d): Olympus cell counter- printed reports: one out of the 20 umbilical cord samples were cultured and passaged successfully until passage 7. (b): cell count report of harvested cells from one culture flask between passage 3 and passage 4, showing viable cell count of 893,000 cells/ml. (c): cell count report of harvested cells from one culture flask between passage 5 and passage 6, showing viable cell count of 4.55 million cells/ml. (d): cell count report of harvested cells from one culture flask between passage 6 and passage 7, showing viable cell count of 3.17 million cells/ml.

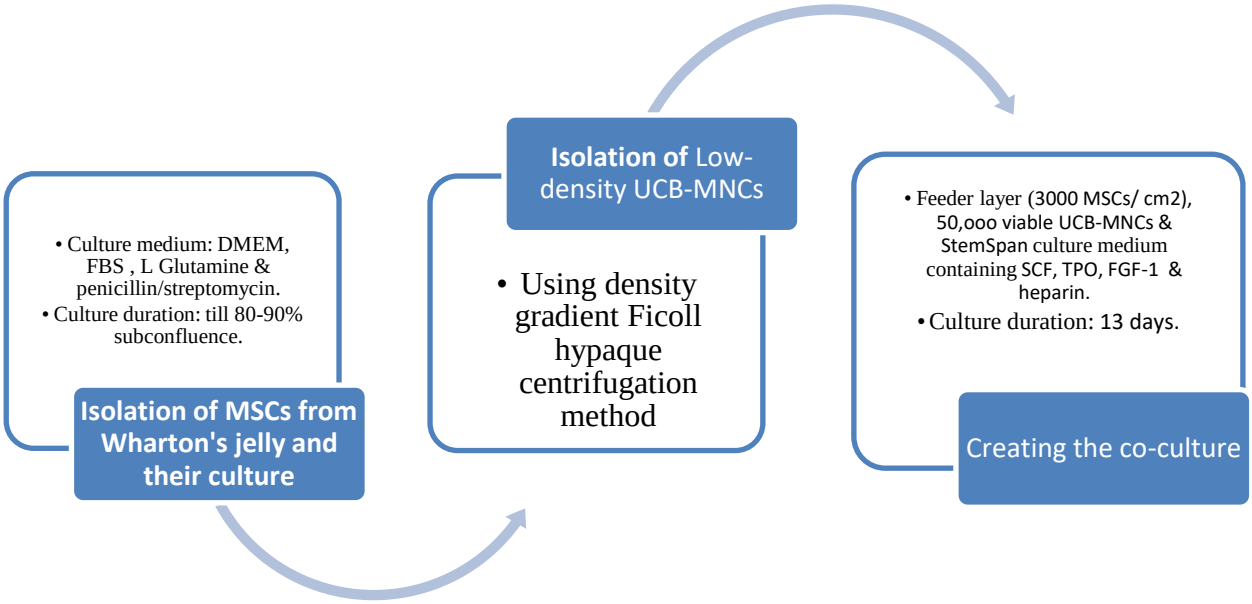


Diagram summarizing culture conditions, ingredients and length of cultures

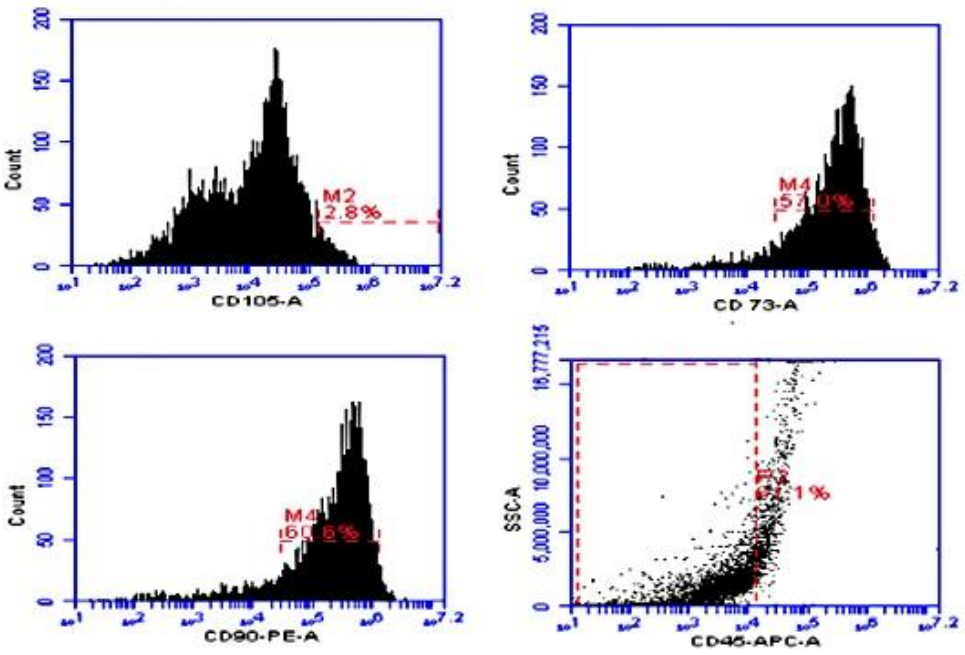


Figure 2. Flowcytometric analysis for a sample of the isolated MSCs: The isolated cells are positive for CD90 (60.6%), CD73 (57%), CD105 (2.8%) and Lack of CD45 surface marker (0.1%)

DISCUSSION

In this study, we created an approach for in-vitro expansion of HSCs from the UCB, aiming to achieve higher cell counts without changing the hematopoietic stem and progenitor cell phenotype and without losing their repopulation ability. Isolation and culture of MSCs from Wharton's jelly were done using the explant method for isolation. We observed that this isolation method was simpler, more economical, and easier to implement than enzymatic digestion protocols. The same observations were shown by Capelli et al.[21], Yoon et al.[22], Hassan et al.[23], and Hendijani[24]. We isolated millions of MSCs by passing the cultures up to passage seven.

We observed significant increases in total cell counts, viable cell counts, and fold change over the culture period. Cell viability was maintained throughout the culture periods, likely attributable to the beneficial expansion of umbilical cord mononuclear cells (MNCs) on the MSC feeder layer. This setup enhanced proliferation rates and reduced apoptosis, consistent with our expectations. Similar results were also reported by Mehrasa et al [14].

HSC expansion cocktails differ across protocols, though most share three core growth factors: stem cell factor (SCF), thrombopoietin (TPO), and either FMS-like tyrosine kinase three ligand (Flt-3L) or fibroblast growth factor-125 (FGF-125)[25].Those three cytokines combinations have been shown to maintain the viability of the HSCs, telomere length, and telomerase activity[26]. They have also been proven to regulate the expression of adhesion molecules and enhance HSC expansion [27].

The use of other cytokines, such as erythropoietin, IL-3, IL-6, IL-11, granulocyte colony stimulating factor (GCSF), platelet-derived growth factor, and macrophage colony stimulating factor, has been more inconstant and has a higher probability of causing cellular differentiation[25]. For instance, IL-3 has been found to support cell expansion in vitro; however, the expanded cells have a reduced in vivo hematopoietic regenerative ability[28].

The source of the MSCs used as feeder layers in our study was cord tissue. Other studies compare the properties of the MSCs isolated from different sources; for example, in the study done by Kadekar et al.[29], they discovered that the placental MSCs were superior to the cord MSCs and were closer to BM MSCs. Moreover, Klein et al.[30] found that UCB and amniotic fluid-derived MSCs were like BM-MSCs and significantly enhanced hematopoietic stem cell (HSC) expansion.

Zhang Y. et al.[31] and Robinson et al.[32] have shown that the mechanism of action of the feeder layers could be through a sustained release of secretory molecules that promote HSC self-renewal, maintenance, and expansion. Da Silva et al.[19] have presented that direct cell-cell contact during the co-culture process has a significant positive impact on the proliferation potential of HSCs. Spees et al.[33] have stated that in MSC coculture protocols, besides direct cell-cell interactions, MSCs secrete microvesicles, exosomes, and growth factors that support MNC proliferation. Melania et al.[34] have observed that Wharton Jelly MSCs possess greater clinical efficacy compared to BM-MSCs. They have assumed that the common anatomical localization of WJ and UCB-HSCs should favor efficient crosstalk between the two types of cells.

In this study, we cultured the isolated MNC fraction without prior enrichment of CD34 or CD133 cells, aiming to achieve significant HSC expansion. We hypothesized that the MSC feeder layer co-culture system would eliminate the requirement for prior enrichment of CD34+ and CD133+ cells. This hypothesis was confirmed in the study by McNiece et al[20].

Our study demonstrated a significant increase in the absolute stem cell count by the end of the 13-day culture period. This suggests that the culture conditions likely preserved the primitive immunophenotype and self-renewal capacity of stem and progenitor cells while minimizing differentiation.

Despite substantial expansion of mononuclear cells (MNCs), the clonogenic potential of cultured progenitor cells was retained and even increased progressively throughout the culture period. The CFU assays performed in this study assessed in vitro granulocyte and macrophage potential but did not evaluate erythroid, megakaryocytic, or platelet lineage potential. To validate our hypothesis, future studies should employ xenograft transplantation

models. Our findings on the clonogenic potential of cultured progenitor cells align with those of Ajami et al.[35], who demonstrated that MSC co-culture systems significantly enhance clonogenic progenitor cell output.

CONCLUSION

This study established a protocol for UCB-MNCs ex-vivo expansion in the presence of Wharton's jelly-derived mesenchymal stem cell feeder layer coculture and with the addition of a combination of growth factors (SCF, TPO, FGF-1, and Heparin). This protocol has resulted in a significant increase in the MNC counts, the absolute stem and progenitor cell counts, the percentage, and the clonogenic function. Therefore, these protocols could enable robust ex vivo expansion of umbilical cord blood mononuclear cells (UCB-MNCs) while preserving the proportion of primitive stem/progenitor cells and their functional capacity. This approach holds significant promise for adult transplantation, particularly in patients lacking matched unrelated donors or requiring urgent transplants.

Limitations

This study had some limitations as indicated below:

1. The small sample size (n = 20) was limited by the high cost of culture media and growth factors, which may restrict the generalizability of the findings.
2. While umbilical cord-derived fibroblasts were isolated, functional validation via differentiation (adipocytes, osteoblasts, and chondrocytes) is still required to fully comply with the ISCT minimal criteria for defining MSCs.
3. In vitro and xenograft transplantation studies in immunocompromised mice (primary and secondary recipients) are needed to assess the long-term engraftment, differentiation potential, and safety of the expanded cells.
4. Systematic controls (e.g., omitting individual components from the culture conditions) are required to identify critical factors driving cell expansion and functionality.

Recommendations: Prior to clinical translation, the following steps are essential:

- Implement sterility testing, viral safety screening, and genetic stability analysis.
- Replace FBS in MSC culture media with alternatives such as serum-free formulations, platelet-rich plasma, platelet lysates, or cord blood serum to eliminate animal-derived components.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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