ISSN: 13434292
Volume 136, Issue 02, December, 2018

Evaluation and improvement of quality of generic pharmaceutical products

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ABSTRACT— During the last three decades hematopoietic stem cell transplantation (HSCT) has become a well-established treatment for many hematologic malignancies. The most important limitation for HSC transplantation is the low number of hematopoietic stem cells (HSC) that can lead to delayed engraftment or graft failures. Numerous attempts have been made to improve in vitro HSC expansion via optimization of various methods such as isolation techniques, supplementing with growth factors, utilizing stromal cells as feeder layer and other culture conditions. This project is aimed to decipher the efficiency of an isolation technique and retrieval of culture expanded HSC from feeder layer using two different harvesting methods. Hematopoietic stem cells from human umbilical cord blood were isolated via MACS mediated $CD34^+$ double sorting. Then, the cells were cultured onto MSC feeder layer for 3 and 5 days. Culture expanded cells were harvested using two different harvesting method namely cell aspiration and trypsinization methods. Hematopoietic stem cell expansion index were calculated based on harvesting methods for each time point. Results: The numbers of HSC isolated from human umbilical cord blood were 1.64×10^6 and 1.20×10^6 cells at single and double sortings respectively. Although the number of sorted cells diminished at the second sorting yet the yield of $CD34^+$ purity has increased from 43.73% at single sorting to 81.40% at double sorting. Employing the trypsinization method, the HSC harvested from feeder layer showed a significant increase in expansion index (EI) as compared to the cell aspiration harvesting method ($p \leq 0.05$). However, the purity of $CD34^+$ HSC was found higher when the cells were harvested using aspiration method (82.43%) as compared to the trypsinization method (74.13%). Conclusion: A pure population of $CD34^+$ HSC can be retrieved when the cells were double sorted using MACS and expanded in culture after being harvested using cell aspiration method.

KEYWORDS: Hematopoietic stem cells, Mesenchymal stem cells, Trypsin, Expansion index, CD34

1. INTRODUCTION

Haematopoietic stem cell (HSC) transplantation has become an inevitable therapy for many blood related disorders. Among all stem cell-based therapies, hematopoietic stem cell transplantation is the only one with a well-established clinical regimen and remains an effective approach for patients with certain haematological diseases [1]. Basically, HSC transplantation involves the intravenous infusion of autologous or allogeneic stem cells collected from bone marrow, mobilized peripheral blood stem cells or umbilical cord blood to the patients [2]. Although bone marrow serves as an ideal source for HSC yet several factors such as very painful invasive procedure, post infections risk, limitation of donor availability and the age of patients often circumvent its wide applications [3]. However, the successful transplantation of umbilical cord blood (UCB) in 1988 has open the gateway to UCB for being considered as an attractive alternative source of HSC in the treatment of haematological malignancies (leukaemia & lymphoma) and non-malignant blood diseases (thalassaemia & sickle cell disease) [4], [5]. Although UCB transplantation has become a big breakthrough in the field of cellular therapy, yet the number of HSC harvested for

cryopreservation or therapy still remains an issue. A substantial number of umbilical cord blood that has been collected by the national and private cord blood banks are discarded due to low number of mononuclear cells which subsequently leads to smaller stem cells fraction. Insufficient numbers of transplantable/storable stem cells barricade the success of clinical and experimental HSC transplantation [6]. Alternatively, numerous attempts have been made to expand HSC derived from UCB in ex vivo via various means such as supplementation with a range of growth factors and basal media; variety of extracellular matrixes; dynamic 3D culture system and utilizing stromal cells as feeder layer [6- 8]. The initial number of $CD34^+$ HSC cells for expansion is a crucial parameter that affects the outcome of the in vitro culture. Besides the optimal culture growth media with mitogenic cytokines, supply of relatively pure population of $CD34^+$ cells for the initial culture escalates the number of expanded $CD34^+$ cells. The physiological condition of HSC in bone marrow requires the stromal compartment as a niche to self-replicate and further differentiate into mature hematopoietic cells [9]. Thus many HSC expansion at laboratory level consumes mesenchymal stem cells as feeder layer to mimic such in vivo condition as MSC in bone marrow niche provide HSC the necessary extracellular matrix and cytokines [10]. However, due to the adhesive nature of HSC and MSC in co-culture system, researchers often face difficulties in separating HSC from feeder cell layer with either a substantial number of HSC retained at the adherent feeder cells or contamination of feeder cells due to forceful harvest. Thus, the present study is aimed to isolate a higher purity of HSC for the initial culture and identify a good harvesting method to collect MSC-feeder layer expanded HSC. This study indicates that a double magnetic activated sorting system (MACS) together with cell aspiration harvesting method yield high fraction of pure $CD34^+$ HSC.

2. MATERIALS AND METHOD

2.1 Samples

Fresh human umbilical cord blood (HCB) was collected from Britannia Hospital, Kajang after obtaining the written informed consent from parents. Approximately 50 to 70 ml of HCB was collected using a commercially available cord blood collecting set which contains 23 ml CPDA-1 (Citrate Phosphate Dextrose Adenine) as an anticoagulant (JMS SINGAPORE PTE LTD). Samples were transported on ice from Britannia Hospital to processing laboratory using a leak-proof container at 16-22 °C. Thirty (30) HCB samples were collected, each experiments were repeated with at least of 5 samples. The approval and ethical clearance from the Faculty of Medicine and Health Sciences (UPM) was attained upon commencement of the study [Reference No: UPM/FPSK/PADS/T7-MJKEtikaPer/F01{Lect_ Sept (08)14}].

2.1 Isolation and culture of HSC

Mononuclear cells were isolated immediately using density gradient centrifugation with Ficoll-Hypaque (Biochrom KG, Berlin, Germany). Briefly, HCB was diluted with 1x PBS prior to layer on Ficoll-Hypaque solution in 50ml centrifugation tube at ratio 1:1. Samples were subjected to the centrifugation of 2000 RPM for 30 min at room temperature without deceleration process. Mononuclear cells were harvested at the interface of plasma and Ficoll- Hypaque and washed once with 1 x PBS. Mononuclear cells were labelled with CD34 monoclonal antibody conjugated with magnetic microbeads. Labelled cells were passed through an affinity column using Auto-MACS system. The column enables a high gradient magnetic separation of CD34 positive selection (Miltenyi Biotec, Bergisch-Gladbach, Germany). Sorted CD34+ cells were subjected to second column sorting to further purify CD34+ HSC. CD34+ HSC were suspended in Iscove's Modified Dulbecco's Media (IMDM) (ThermoFisher Scientific, USA) at a density of $2x10^4$ cells/well, together with 1% PenStrep (ThermoFisher Scientific, USA), 0.5% Fungizone (ThermoFisher Scientific, USA) and 10% foetal bovine serum (FBS) (ThermoFisher Scientific, USA). HSC suspensions were seeded

on 24-well plates at a density of $2x10^4$ cells/cm^2 on top of a confluent Mitomycin C treated (Sigma) (0.5 mg/ml solution prepared in IMDM+10% FBS) MSC layer. Cells were incubated in a 37°C and 5% CO2 humidified incubator, for 3, and 5 days, without medium change.

2.3 Flow cytometer analysis

The purity of CD34+ HCS was assessed by flow cytometry using allophycocyanin (APC)-conjugated anti-human CD34 monoclonal antibody according to the manufacturers' protocol. Briefly, 10^5 to 10^6 of HSC were stained with 5 µl anti-human monoclonal antibodies (CD34-APC and CD45- PerCP-Cy5.5) for 20 minutes at 2-8ºC. Then HSC were washed with 1x PBS and immediately analysed using FACSS Fortessa (BD Biosciences, San Jose, CA, USA) flow cytometer. A total of 10,000 events were recorded and data were analysed with BD FACSDiva Software V.6.1.1 (BD Biosciences, San Jose, CA, USA). An additional staining with propidium iodide (PI) was performed to allow exclusion of dead cells. Hematopoietic stem cells are identified based on CD34high CD45low population in dot plot. The positivity of any given antibody stain was determined by quadrant analysis as compared to the isotypic negative controls (cells stained with the isotype controls for, PerCP-Cy5.5 Mouse IgG1 and APC Mouse IgG1 were used as negative controls).

3. RESULTS

The number of viable CD34+ HSC after the single and double sorting was manually counted using hemocytometer under the phase contrast microscope. Viability of cells was determined by trypan blue exclusion test where the nucleus of dead cells stained with trypan blue. The mononuclear cells from cord blood were subjected for MACS CD34 positive selection with initial cell numbers of 172×106/3ml. Upon single and double sorting, the number of CD34+ HSC collected were 1.64×106 and 1.20×106 respectively (Figure 1A). The second enrichment process had lowered the number of sorted cells to 70% of initially sorted cells (single sorting). After the single and double sorting process, the percentage of cells that expressing CD34 marker were 43.73% and 81.40% respectively.

4. DISCUSSION

Due to its inevitable therapeutic value, hematopoietic stem cells (HSC) have become major stem cells that have been commonly used in many haematology related transplantations. Although the early establishment of HSC and initial clinical uses started with bone marrow, later other post-natal and peri-natal tissues such as peripheral blood and umbilical cord blood were identified as potential source for HSC. Among all, umbilical cord blood derived HSC serves as an ideal source for stem cells because of less ethical concern, abundantly available as post-delivery waste products and the primitive nature of HSC. Two common methods that have been widely consumed to isolate HSC are fluorescent-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) using a single or an array of specific antibodies [11], [12]. In the present study, $CD34^+$ HSC were sorted using monoclonal anti-CD34 antibody conjugated with magnetic microbeads. Although, there are several surface markers being used as of single or in combination to sort HSC, yet the use of CD34 has been the utmost preference in isolating and characterizing HSC. In comparison to FACS, MACS method is much feasible and affordable. Sorting technique using magnetic microbeads conjugated antibody allows an aseptic isolation of desired cell population as this could easily performed in cell culture biosafety cabinet. Despite the multiple markers mediated isolation and identification of target population by flow cytometer which sanctions much specific isolation, yet this method require a highly trained personnel and often not fully warrant the aseptic sorting. Since the frequency of HSC in human umbilical cord blood is very low as reported approximately 0.1–4.9 %, [13] our data showed that approximately 0.66 % of umbilical cord mononuclear cells are CD34+ HSC as we could isolate $1.20×10^6$ CD34+ HSC from 170.20 $x10^6$ mononuclear cells. Employing double sorting method, we

have also demonstrated that the purity of separated cells is 81.4% when 50-70 ml of human umbilical cord blood was consumed.

5. CONCLUSION

The present study showed that the double sorting technique using magnetic separation yields the required CD34+ HSC population for in vitro expansion. The expanded HSC on MSC feeder layer can be optimally harvested using cell aspiration method to maintain high purity of HSC.

6. ACKNOWLEDGMENTS

This work is supported by Putra Grant-Postgraduate initiative (GP-IPS/2014/9432500 from Universiti Putra Malaysia, Malaysia.

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ISSN: 13434292
Volume 136, Issue 02, December, 2018

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