Agitation increases expansion of cord blood hematopoietic cells and promotes their differentiation into myeloid lineage

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Received: 10 March 2014 / Accepted: 2 February 2015 / Published online: 12 August 2015
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Abstract Mechanical stress caused by agitation is one of the factors that can affect hematopoietic stem cell expansion in suspension bioreactors. Therefore, we have investigated the effects of agitation on umbilical cord blood hematopoietic stem cell (UCB-HSC) growth and differentiation. A comparison was made between various agitation rates (20, 40 and 60 rpm) in spinner-flask and cells cultured in glass petri dish as a static culture. Moreover, the fluid dynamic at various agitation rates of spinner-flask was analyzed to determine shear stress. The spinner-flask contained a rotational moving mixer with glass ball and was kept in tissue culture incubator. To reduce consumption of cytokines, UCB-serum was used which widely decreased the costs. Our results determined that, agitation rate at 40 rpm promoted UCB-HSCs expansion and their colony forming potential. Myeloid progenitors were the main type of cells at

40 rpm agitation rate. The results of glucose consumption and lactic acid production were in complete agreement with colony assay and expansion data and indicated the superiority of culture in spinner-flask when agitated at 40 rpm over to other agitation speeds and also static culture. Cell viability and colony count was affected by changing the agitation speed. We assume that changes in cell growth resulted from the effect of shear stress directly on cell viability, and indirectly on signaling pathways that influence the cells to differentiate.

Keywords Agitation rate · Hematopoietic stem cell expansion · Spinner flask · Suspension culture

Introduction

For the first time in 1989 the transplantation of hematopoietic progenitor cells derived from umbilical cord blood (UCB) was accomplished (Gluckman et al. 1989). Then, because of its availability, ease of gathering and low likelihood of graft versus host diseases, UCB-hematopoietic stem cells (UCB-HSCs) have been broadly applied to treat hematologic disorders, immune deficiency diseases and genetic diseases (Almici et al. 1995; Andrade-Zaldívar et al. 2008; Kowalczyk et al. 2011). Regardless of all advantages mentioned, low count of stem cells per each unit of UCB restricts its application especially for

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adults (Andrade-Zaldívar et al. 2008; Cabrita et al. 2003). Consequently, employing methods for ex-vivo expansion of stem cells seems essential (Andrade-Zaldívar et al. 2008; Kowalczyk et al. 2011; Broxmeyer et al. 2006). Traditionally, static culture systems such as T-flasks, plates and gas-permeable culture bags are utilized for ex vivo expansion of HSCs (Cabral 2001; Kowalczyk et al. 2011). However, traditional culture techniques have many limitations. Firstly, absence of mixing causes high concentration gradients in dissolved oxygen, pH, cytokines and metabolites. Secondly, frequent handling to exchange culture media increases the probability of contamination. Thirdly, there are troubles related to control and on-line monitoring of significant culture parameters in static cultures. Finally, there is a limitation in cell productivity of static cultures because of low surface area available (Collins et al. 1998b; Nielson 1999; Yang et al. 2008). To overcome deficiencies in static cultures and remove difficulties related to non-homogeneity of the culture and mass transfer, different kinds of bioreactors such as stirred-tank, packed-bed, rotating-wall vessel and perfusion-chamber have been invented to expand stem cells (Liu et al. 2006; Meissner et al. 1999; Palsson et al. 1993).

The type of mixer and mode of its movement, its diameter, geometry of vessel, bubble formation and agitation rate, all are important features of bioreactors that affect cell culture's efficiency and also cell surface markers expression especially in suspension cultures (Gilbertson et al. 2006).

Since the first bone marrow hematopoietic stem cell (BM HSC) expansion in suspension culture was performed (Zandstra et al. 1994), various studies about HSCs expansion in stirred cultures have been accomplished due to complexities related to bioreactor cultures. To date, proliferation and differentiation potential of mononuclear cells (MNCs) derived from different sources in diverse suspension cultures have been investigated and comparisons between static and dynamic cultures have been made (Collins et al. 1998a; Kwon et al. 2003; Shayan et al. 2012; Yang et al. 2008). However, the accurate influence of shear stress on UCB–MNCs expansion and their functional characteristics still remains undetermined. Therefore, a spinner-flask equipped with a rotational moving mixer with glass ball was used to investigate the effect of agitation rate on HSCs expansion and differentiation. The results were compared with results from static culture.

Materials and methods

Cell separation procedures

UCB samples were obtained from Royan Public cord blood bank (Tehran, Iran). UCB–MNCs were isolated using density gradient of lymphodex (Inno-train, Shirley, Solihull, UK) by centrifugation at 1,000g for 25 min at 25 °C. In order to remove existing red blood cells (RBCs) in collected intermediate layer, the RBC lysis buffer (0.15 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) was added, mixed very well and then washed with phosphate buffer saline. The resultant cells were suspended in the cell culture medium at density of 10⁶ cells/ml in T-150 flasks (Falcon, Corning, Tewksbury, MA, USA) and incubated for 24 h at 37 °C in a humidified incubator with 5 % CO₂, before being entered into either suspension or static culture systems. This pre-incubation was done to accustom the cells to the culture conditions before facing high damages resulted from fluid mechanics (Collins et al. 1998a; Liu et al. 2006).

Cell culture condition

Iscove's modified Dulbecco's medium (IMDM) (Sigma, St. Louis, MO, USA) included 10 % (v/v) fetal bovine serum (FBS) (GIBCO, USA), 10 % (v/v) cord blood serum (CBS), 50 U penicillin/ml (Gibco, Invitrogen, Darmstadt, Germany) and 50 µg streptomycin/ml (Gibco, Germany). The culture medium also included low doses of purified recombinant human cytokines: 5.33 ng interleukin-3 (IL-3)/ml, 16 ng stem cell factor (SCF)/ml, 2.13 ng granulocyte-monocyte colony stimulating factor (GM-CSF)/ml and 7.47 ng flt3 ligand (FL)/ml (R&D Systems, Minneapolis, MN, USA) (Liu et al. 2006; Yao et al. 2003). Cells which were cultured in cytokine free medium were used as negative control.

Suspension culture was performed in a 100 ml spinner-flask with a single glass-ball stirring pendulum (CELLSPIN system, Integra Biosciences, Hudson, NH, USA) with 50-ml culture medium equipped with a rotational moving agitator at 20, 40 and 60 rpm rotational rates (Fig. 1). The static culture was conducted in a 20-ml glass petri dish (BD biosciences, Franklin Lakes, NJ, USA). Prior to cell culture, the surface of all vessels and the dishes were coated by silicone (Sigma) in order to prevent the cells from attaching to the surface. Both spinner-flask and petri dish were placed in a

Fig. 1 Schematic diagram of Spinner-flask (a, b)

humidified incubator with 5 % CO₂ and maintained at 37 °C. An inoculum density (ID) of 106 cells/ml was used for both culture systems. The medium was exchanged every 2 days, by removal of 50 % (v/v) of the cell suspension, centrifugation at 1,000g for 10 min, removal of the waste medium and returning the cells with fresh equal medium to the system.

Cell growth and cell type assays

Cells were collected on days 0, 3, 7 and 14; to investigate the influences of culture type and agitation speed on cell growth and differentiation. Total cell counts and viability were evaluated manually using 0.4 % trypan blue. To assess cell differentiation, colony-forming and immunophenotyping assays were carried out at various time intervals during culture period.

For colony-forming assay, 1×10^4 cells were harvested and added directly to 2 ml semisolid IMDM containing MethoCult SF4436 (Stemcell Technologies, Vancouver, BC, Canada) in 35 mm-gridded dishes (Nunc International, Roskilde, DK). After 14 days of incubation in fully humidified conditions and atmosphere of 5 % CO₂ at 37 °C, colonies including more than 50 cells were scored under an inverted microscope (Olympus, Tokyo, Japan).

For immunophenotyping, 10^6 cells were collected and analyzed by two-color flow cytometry on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34, phycoerythrin (PE)-conjugated anti-human CD133, anti CD38 (FITC) and anti CD90 (PE) antibodies (all from BD-Pharmingen, Franklin Lakes, NJ, USA). A replicate sample was stained with FITC-mouse IgG1 and PE-mouse IgG1 as an isotype control to ensure specificity.

Glucose consumption and lactic acid production

Glucose and lactic acid concentration on various culture days were determined by high performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) equipped with a computer system to run and control all the calculations for the instrument. A sugar pak-1 column (300 × 6.5 mm) (Waters) was used in this study, mobile phase was deionized water with the flow rate of 0.6 ml/min and the temperature of the oven was optimized to 75 °C. The concentrations of glucose and lactic acid were assessed from the peak area under the curve using Empower software.

Model assumptions

Based on impeller speed and calculated Reynolds numbers we assumed a turbulent flow regime in the vessel. The fluid is Newtonian and possesses incompressible flow characteristics. The geometry of the spinner flask is shown in Fig. 1. All the dimensions of vessel and impeller were measured from the real object and the total volume of the fluid body in the model was set as 50 ml for the simulation of 100-ml spinner flask. The fluid volume in the vessel was 50 ml, and the vessel diameter was 6 cm.

Model equations

The continuity and Navier–Stokes equations are used to describe the flow in the spinner flask: Eq. 1 (continuity)

$$(\nabla \cdot \bar{V}) = 0 \quad \text{and} \quad (\nabla \cdot V') = 0 \quad (1)$$

Eq. 2 (Navier–Stokes)

$$\frac{\partial}{\partial t} \rho \bar{V} = -\nabla \bar{P} - [\nabla \cdot \rho \bar{V} \bar{V}] - \left[\nabla \cdot \left(\bar{\tau}^{(v)} + \bar{\tau}^{(t)} \right) \right] + \rho g \quad (2)$$

where ρ is the density of the fluid, $\bar{V}$ is mean velocity or time-smoothed velocity, V' is velocity fluctuations. Also $\bar{P}$ is time-smoothed pressure, and $\bar{\tau}^{(v)}$ is time-smoothed viscous momentum flux and $\bar{\tau}^{(t)}$ is turbulent momentum flux tensor.

Computational details and model parameters

The fluid dynamic simulation of spinner-flask's glass balls was carried out when agitated at various agitation

rates. The spinner flask was modeled by discretizing the physical domain into 293,252 tetrahedron cells by Gambit meshing software. Based on statistical analysis aforementioned cell numbers were adequate to achieve a mesh independent solution. The fluid dynamic simulation of mixing in spinner-flask was carried out when agitated at various agitation rates. Commercial CFD software package fluent (Version 6.3, ANSYS) was used to perform numerical calculation of the velocity field in the spinner flask.

Statistical analysis

Experiments were repeated at least three times ($n = 3$) and the results were presented as the mean $\pm$ standard deviation (SD). The significance of differences was determined using one-way ANOVA and expressed with $P < 0.05$.

Results

Expansion of UCB–MNCs in static and suspension cultures

UCB–MNC in both culture types was seeded at density 1×10^6 cells/ml, and cultured for 14 days. The presented results revealed that expansion profiles of all culture systems were relatively similar until day 3 which had a downward trend. Then during extra 11 days of culture, the total cell density increased in static culture ($2.38 \times 10^6 \pm 0.037$ cell/ml) and spinner flask at the rotational speeds of 20, 40 and 60 rpm ($3.063 \times 10^6 \pm 0.057$, $5.32 \times 10^6 \pm 0.059$ and $3.33 \times 10^6 \pm 0.193$ cells/ml, respectively) which was significantly prominent at the agitation rate of 40 rpm (Fig. 2a, b). Evaluation of cell viability determined that the most viable cells were observed at 40 rpm agitation. Meanwhile, the cell death increased at higher (60 rpm) and lower agitation speed (20 rpm) and static culture. The differences between static culture and 20 rpm agitated group was not significant ($P > 0.05$) (Fig. 2c). The cell numbers decreased significantly in negative controls that was feed just by cytokine free medium (Fig. 2a). The growth reduction in negative controls was related to decreased viability both in spinner flask (16 ± 1.9 %) and static culture (5.7 ± 1.47 %) 14 days post culture (Fig. 2c).

Glucose consumption and lactic acid production confirm the results of cell growth. Actually, glucose concentration decreased in all culture systems during the culture period, and reduction was prominent at day 7, and then increased slightly (Fig. 2d). Instead, the lactic production augmented in all culture conditions with a maximum peak at day 7 and then slowly decreased (Fig. 2e). The metabolic reaction was maximum in spinner flask at agitation rate 40 rpm (3.28 times reduction in glucose and 33.3 times augmentation in lactic acid compare to static culture) (Fig. 2d, e).

Computational fluid dynamic (CFD) analysis in spinner-flask

A key parameter of spinner flasks to control is the agitation forces, which should be sufficient to ensure homogeneous distribution of cells, nutrients and oxygen, but not too severe to harm the cells. Velocity profile and the shear stress distribution in the vessel are among major parameters that should be considered in bioreactor design for suspension culture. In order to decrease the shear stress we used a spinner flask equipped with pendulum-shaped (glass-ball) impeller. The computational fluid dynamic analysis at agitating speeds of 20, 40 and 60 rpm was performed. Velocity profiles resulted from CFD analysis is shown in Fig. 3. The optimal value for shear stress is different for various stem cell subtypes (Rodrigues et al. 2011). The threshold shear stress that has been reported to be detrimental to HSCs is 0.092 Pa (Ma et al. 2007). The shear stress in an agitated vessel varies within the fluid. The maximum shear stress occurs at the impeller blade tip. Although the fraction of the fluid with maximum shear stress is very small, it can cause cell damaging. As a result it is important to calculate maximum shear stress in addition to average shear stress on the impeller (Gilbertson et al. 2006). The minimum, average and maximum wall shear stress at agitation rates of 20, 40 and 60 rpm in spinner flask was calculated, and the results are shown in Table 1. Also by the use of CFD it was calculated that the percentage of impeller surface that experience a wall shear stress higher than the threshold value for HSCs at 20, 40 and 60 rpm are 4.79, 40.51 and 93.71 %, respectively.

As indicated in Fig. 3, the fluid velocity was lower at 20 rpm that caused insufficient mixing to create a

Fig. 2 **a** Total cell density, **b** expansion folds of total nucleated cells, **c** percentage of viable cells, **d** glucose concentration and **e** lactic acid concentration in cultured cells in spinner-flask at different agitation rates and static culture. Data are the mean $\pm$ SD of three separate experiments. * $P < 0.05$

uniform environment, therefore HSCs expansion was poor (Fig. 3a). Uniform mixing was observed at agitation rate of 40 rpm (Fig. 3b). The fluid velocity around the impeller was the highest at 60 rpm that caused to augment shear stress at the impeller tip (Fig. 3c). This level of shear stress resulted in lower HSCs expansion and higher cell death.

Effect of agitation on HSCs differentiation

The main question in this study was whether agitation affects cell stemness characteristics and induces cell differentiation? To test our hypothesis; colony-

forming assays and immunophenotyping were carried out. The results of colony-forming assay which represents function of hematopoietic stem/progenitor cells showed that the number of granulocyte-macrophage colony forming unit (CFU-GM) and erythroid-granulocyte-macrophage colony forming unit (CFU-Mix) and colony forming count (CFC) increased in the spinner flask at an agitation of 40 rpm (Fig. 4a-c).

The number of CFU-GM enhanced until day seven in all culture systems, then reduced during the culture (Fig. 4a). The number of CFU-Mix raised during the first 3 days of culture in all systems, and for the rest of the culture a descending trend was observed (Fig. 4b).

Fig. 3 Illustration of cell Reynolds number calculated in CFD analysis of 100-ml glass-ball spinner flask. Within the performed agitating speed, **a** 20 rpm, **b** 40 rpm, **c** 60 rpm, the

distribution of the cell Reynolds number was the same, only with variant scale of values

Table 1 Minimum, maximum and average shear stress at agitation rates of 20, 40 and 60 rpm

Agitation speed (rpm)	Minimum wall shear stress (Pa)	Average wall shear stress (Pa)	Maximum wall shear stress (Pa)
20	0.0016	0.041	0.14
40	0.0054	0.109	0.35
60	0.01	0.19	0.62

Fig. 4 The number of CFU-GM (a), CFU-Mix (b) and CFCs (c) in spinner-flask at different agitation rates and static culture. Data are the mean $\pm$ SD of three separate experiments. * $P < 0.05$

At the end of the culture, the average expansion of colony forming cells (CFCs) in static culture and spinner-flask at the agitation rates of 20, 40 and 60 rpm was 19.22 ± 3.54 , 26.67 ± 3.86 , 59.67 ± 9.84 and 32.33 ± 0.47 , respectively (Fig. 4c).

To confirm above mentioned results; the percentage of HSCs “CD34⁺CD133⁺ and CD90⁺CD38[−]” and their commitment progenitor “CD90[−]CD38⁺” was determined by flow cytometry (Fig. 5). The results revealed that the number of CD34⁺CD133⁺ decreased suddenly until day 7 and during the culture time in all culture systems. At the end of the culture time, the lowest reduction was seen at 40 rpm ($P < 0.05$) and the highest was at 20 rpm of agitation and static culture ($P < 0.05$) (Fig. 5a). Although the number of CD90⁺CD38[−] decreased in all groups but there were not any significant differences between groups ($P \geq 0.05$) (Fig. 5b). Conversely, the number of CD90[−]CD38⁺ increased in the first 7 days of culture followed by stability for the rest of the period for all culture systems (Fig. 5c). The percentage of CD90[−]CD38⁺ was prominent at 20 rpm agitation speed. However, the differences were not significant between test groups and static culture.

Discussion

Recently clinical applications of UCB-HSC have increased; therefore development of efficient tools to expand HSCs is necessary. Spinner flasks or stirred bioreactors have been used for robust expansion of HSCs, because they do not require surface attachment to grow (Zandstra et al. 1994; De León et al. 1998; Ratcliffe et al. 2012). Agitation is one of the important parameters in stirred bioreactors or spinner flasks that may cause unexpected cell death (Boehm et al. 2010) or differentiation (Hunt et al. 2014). There are few reports to evaluate effect of agitation on HSCs differentiation (Jing et al. 2013); therefore, here we demonstrate the effect of different agitation speeds of spinner flask on UCB-HSCs expansion and differentiation.

Non purified MNCs derived from cord blood were used to mimic in vivo environment, where HSCs close to progenitor cells and partially differentiated cells. Progenitor cells provide exogenous factors to improve cell expansion (Scadden 2006; Collins et al. 1998a). On the other hand, we used UCB serum to provide appropriate growth factors for UCB-HSCs expansion in combination with IL-3, SCF, GM-CSF, FLt3-L

Fig. 5 **a** Percentage of CD34⁺CD133⁺ cell, **b** CD90⁺CD38[−] cell and **c** CD90[−]CD38⁺ in static culture and spinner-flask at different agitation rates. Data are the mean $\pm$ SD of three separate experiments. * $P < 0.05$

based on our previous experiment (Shayan et al. 2012). Use of non-purified cells, UCB-serum and cytokine combination decreased the cost of experiments.

According to our data, expansion profiles of UCB-MNCS in both static and spinner flask culture were relatively similar. They reduced until day 3 then cell numbers increased until day 14. Among all culture systems, spinner flask with agitation rate 40 rpm showed robust cell expansion (about 5 fold increase in total cell number and colony forming units) which was because of maintaining the cell viability and appropriate metabolic reaction (Glucose consumption/Lactic production). Interestingly, the metabolism reaction was increased in the first 7 days (the same time to increase CFU-GM) and then slightly decreased.

Considering the current literature, HSCs expansion in our study was lower than in other reports (Luni et al. 2011; Choi et al. 2010; Jing et al. 2013), which is mainly related to the type of the cells, mixer type and feeding protocols. Most of the studies have used purified CD34 or CD133 cells (Luni et al. 2011; Choi et al. 2010; Jing et al. 2013), that enter easily into mitosis in the presence of cytokines and therefore increase vastly the yield of expansion (Du et al. 2014). In our study most of the differentiated cells lost in first 3 days and then remaining cells that were mainly

HSCs and early progenitor cells (data not shown) started to proliferate until day 7.

Acceptable level of shear stress is a critical parameter in suspension cultures of mammalian cells (Gilbertson et al. 2006). This characteristic is affected by vessel geometry, impeller design, impeller speed, and addition of equipment such as probes and sparging devices. With the assumption that the shape of impeller affects HSCs differentiation we used a glass ball mixer with pendulum mixing pattern to decrease shear stress, this is while other researchers have used common types of mixers like paddle blade impeller and spiral discs.

The worst expansion in static culture or spinner flask agitated at 20 rpm is related to absence or insufficient mixing; that generates a non-homogenous environment and causes concentration gradients in nutrients, metabolites and cytokines (Choi et al. 2010).

It is expected to gain more expansion by increasing the agitation rate to higher amounts because of improvement of homogeneity in the system. This prediction was true until increasing agitation rate to the amount of 40 rpm. However, at 60 rpm agitation speed we suddenly faced lower cell expansion in comparison to 40 rpm. This is because of increasing wall shear stress at such high agitation speeds that has

caused harm to the cells (O'Connor and Papoutsakis 1992; Sucosky et al. 2004). As mammalian cells are very shear sensitive, cell damage because of mixing is very common in suspension cultures. High amount of shear stress may result in cell death (Gilbertson et al. 2006; Papoutsakis 1991). Based on the results of CFD analysis, at all agitation speeds of 20, 40 and 60 rpm a portion of the mixer surface experience higher shear stress than the threshold shear stress of HSCs (0.092 Pa). This is the reason that cell viability is not 100 % even at the most appropriate cell culture conditions in our study. However at 40 rpm, the percentage of mixer surface that faces a higher shear stress than threshold value is significantly less than this percentage at 60 rpm agitation rate. This is the most important reason of having higher cell expansion at 40 rpm agitation rate in comparison to 60 rpm. Also, by interpolating the data from CFD analysis we have found out that at the agitation speed of 36 rpm, the average amount of wall shear stress is exactly lower than the threshold shear stress for HSCs (0.092 Pa). Therefore, studying cell expansion and differentiation characteristics of cells cultured at 36 rpm agitation rate seems interesting for future studies.

Stirring the suspension culture results in the formation of spatial differences in the pattern of liquid flow, leading to cell damage caused by hydrodynamic shear stress (Chisti 2000; Gregoriades et al. 2000). Cell death at high shear is caused by the applied shear force exceeding the cell bursting force, whereas the damage caused at low shear stress is mainly due to the papillated state of the cell, something that occurs when the turgor pressure pushes the cytoplasm out through transient pores formed in the cell (Mardikar and Niranjana 2000). Also, the susceptibility of cells to hydrodynamic damage can vary as they move through the cell cycle, with larger cells in S and G2 phase are more prone to be damaged than G1 cells, presumably due to their size (Brindley et al. 2011).

We could not compare calculated fluid dynamic with similar studies, to find out which of them provide better expansion, lower differentiation with lower shear stress. Most of the studies just reported the expansion profile, colony formation without calculation of shear stress. Based on flow cytometry analysis and colony forming assay, we determined that agitation induced HSCs differentiation. The number of CFU-GM enhanced until day seven in all culture systems, and then decreased during the culture in trend

with metabolic reaction. The number of CFU-Mix augmented during the first 3 days of culture and for the rest of the culture a descending trend was observed. Therefore HSCs may rise in early culture time as reported earlier (Jing et al. 2013) then differentiate to early progenitor cells specially $CD90^{-}CD38^{+}$ of early myeloid progenitors.

In conclusion; the principal of further studies is to determine the influence of other parameters such as shape of impeller and its direction on HSCs differentiation and more clinical trials must be conducted for guarantying the safety.

Acknowledgments The authors thank Mr. Fazel S. Sahraneshin for his excellent technical assistance and helpful advices at flow cytometry data. This study was funded by a grant provided from Royan Institute.

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

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