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Short communication

Maximizing the *ex vivo* expansion of human mesenchymal stem cells using a microcarrier-based stirred culture system

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ABSTRACT

Bioreactor systems have been developed as alternatives to standard culture flasks due to their homogeneous nature, easiness of monitoring and increased cell production. Here we investigated the *in vitro* expansion of bone marrow (BM) mesenchymal stem cells (MSC) in spinner flasks, using gelatin microcarriers (*Cultispher S*) to support cell adhesion and proliferation. MSC expansion was performed using a low-serum containing medium (2% of fetal bovine serum, FBS).

A strategy was defined for the maximization of cell expansion: microcarriers were pre-coated with FBS in order to increase cell seeding efficiency and an adequate feeding regime was established (25% medium exchange everyday). The maximum cell density, 4.2×10^5 cells/mL, was obtained at day 8, corresponding to a fold increase in total cell number of 8.4 ± 0.8. Expanded MSC retained their differentiation potential into adipogenic and osteogenic lineages, as well as their clonogenic ability. Harvested cells expressed >90% of CD73, CD90 and CD105 markers.

These results demonstrated that a microcarrier-based stirred culture system is adequate for human MSC expansion, using a low-serum containing medium, allowing the generation of significant cell numbers for potential applications in regenerative medicine.

1. Introduction

Mesenchymal stem cells (MSC) are currently exploited in numerous clinical trials to investigate their potential in immune regulation, hematopoiesis and tissue regeneration (Caplan, 2007). Currently applied doses are in 1–5 millions MSC/kg body weight range (Subbanna, 2007), thus a fast and reliable *ex vivo* expansion method is needed to meet the highly demanding cell dose.

Bioreactor systems have been developed as alternatives to standard flasks for *in vitro* culture (Cabrita et al., 2003; King and Miller, 2007). Simple stirred vessels, spinner flasks, offer attractive advantages over other culture configurations such as a ready scalability and higher homogeneity minimizing concentration gradients (pH, dissolved oxygen, metabolites), especially when combined with microcarrier culture which is one of the most effective techniques to immobilize and cultivate adherent cells under stirred conditions (Abranches et al., 2007; Fernandes et al., 2007; Levine et al., 1977). Several attempts have been made to expand MSC from different species in spinner flasks using microcarriers: porcine MSC (Frauenschuh et al., 2007), goat MSC (Schop et al., 2008) or rat MSC (Sart et al., 2009). In addition, one single report on the expansion of human MSC using microcarriers in a spinner flask was published (Wu et al., 2003).

In these studies we demonstrated the feasibility of using a microcarrier-based stirred culture system for the successful expansion of human BM MSC, using a low-serum containing medium.

2. Results and discussion

2.1. Feasibility of using a low-serum content medium (MesenPRO RS™) for the successful expansion of BM MSC

The expansion of mesenchymal stem cells (MSC) in clinical trials has been performed using a culture medium supplemented with selected lots of fetal bovine serum (FBS). However, ideally, the clinical-scale propagation of MSC should use a highly standardized animal protein-free fully defined medium (Shahdadfar et al., 2005; Bernardo et al., 2007; Schallmoser et al., 2007; Shetty et al., 2007). Here we propose the use of a medium containing 2% of FBS and the conclusions taken from the operation of this system will provide important insights in a near future towards the implementation of more suitable and safe medium formulations.

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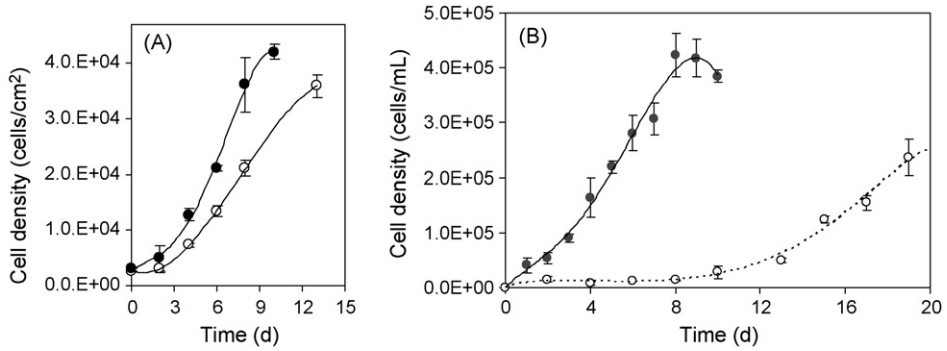

Fig. 1. (A) MSC growth under static conditions (polystyrene culture flasks, 3000 cells/cm^2, medium exchange every 4 d) in (●) MesenPRO medium or (○) DMEM 10%FBS; Error bars represent SEM of two independent experiments (donor #1). (B) MSC expansion in spinner flasks using: (○) 1 g/L *Cultispher S*, 25% medium exchange each 2 d, 50,000 cells/mL inoculated. Error bars represent SEM of two samples (donor #1); and (●) 2 g/L *Cultispher S*, preincubated in FBS prior to inoculation, 25% medium exchange everyday (from day 3 onwards), 50,000 cells/mL inoculated. Error bars represent SEM of three independent experiments, *i.e.* using cells from three different donors.

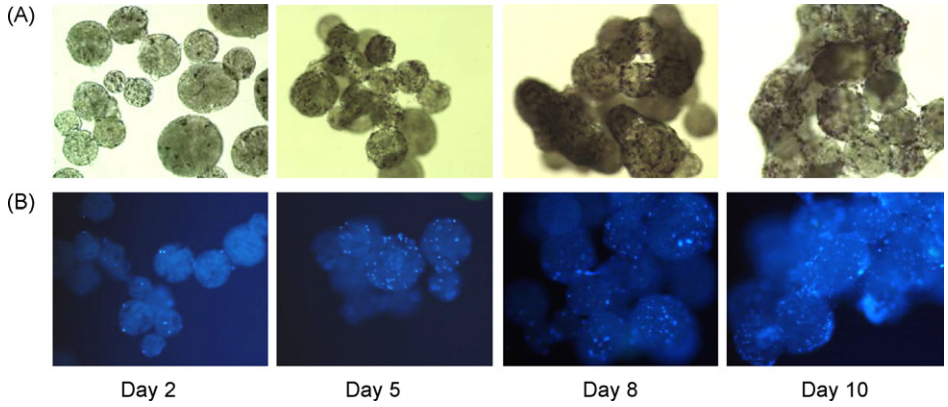

Fig. 2. MTT (A) and DAPI (B) stainings of *Cultispher S* beads during the time of culture in spinner flask were performed according to Fernandes et al. (2007).

Firstly we performed a side by side comparison of BM MSC *ex vivo* expansion using the traditionally used Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (dos Santos et al., 2010) and MesenPRO RS^{TM}. These results are shown in Fig. 1A. MSC expanded faster in MesenPRO RS^{TM} ($\mu = 0.36\,d^{-1}$, $r^2 = 0.79$) than in DMEM 10% FBS ($\mu = 0.32\,d^{-1}$, $r^2 = 0.98$), and the maximum density, nearly 4.0×10^4 cells/cm^2 was attained earlier (at day 10). Importantly, the expansion under low-serum content medium did

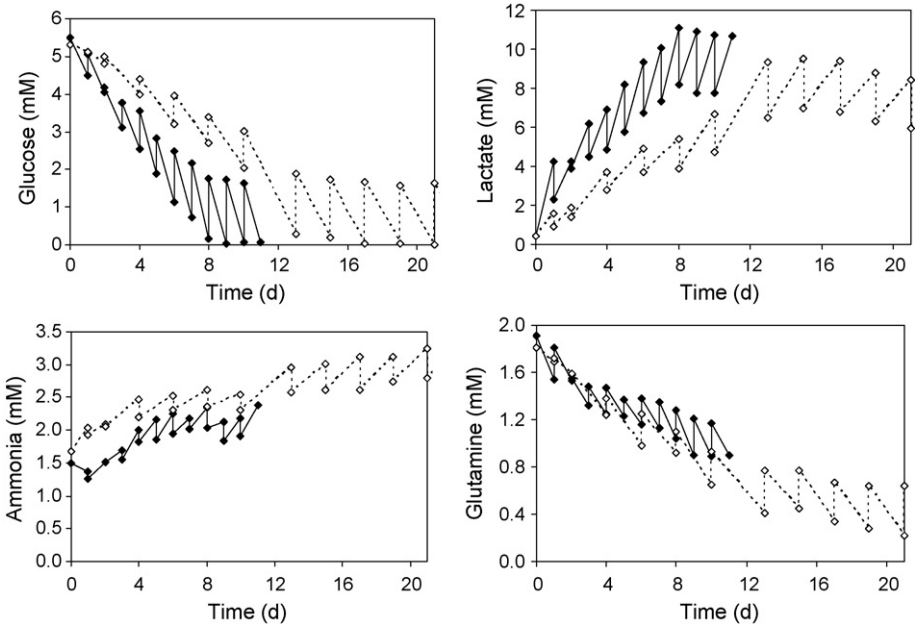

Fig. 3. Metabolic profiles during the culture of MSC in feeding regime 1, (○): 25% of the medium was changed each 2 d; and feeding regime 2, (●): 25% of the medium was changed everyday from day 3 onwards; *Cultispher S* microcarriers were preincubated in FBS prior to inoculation. Cells from donor #1 were utilized in both experiments.

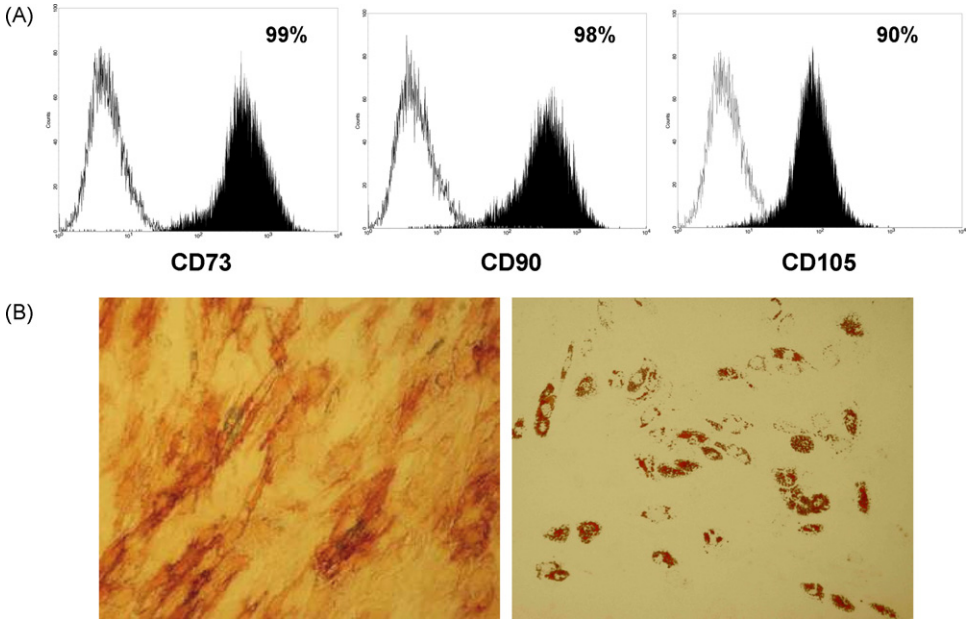

Fig. 4. Characterization of harvested cells after the expansion in spinner flask using MesenPRO^{TM} medium under optimized conditions. Flow cytometric analyses and multilineage differentiation assays (osteogenic and adipogenic) we performed as previously described (dos Santos et al., 2010). (A) Immunophenotyping; (B) differentiation into osteogenic and adipogenic lineages.

not alter the characteristic immunophenotypic cell profile, nor the multilineage differentiation ability and clonogenic potential (data not shown).

2.2. BM MSC expansion in a microcarrier-based spinner flask culture system

Based on our preliminary studies, two key factors for the success of our culture system were identified: (i) initial cell adhesion to the microcarriers; and (ii) feeding regime.

2.2.1. Enhancing initial cell adhesion by microcarrier pre-coating with FBS

Cell seeding under serum-free or low-serum conditions is the most critical stage in microcarrier culture. In our preliminary experiments using MesenPRO RS^{TM} to support the expansion of BM MSC in a spinner flask, less than one-third of the cells adhered efficiently to the microcarriers which resulted in a long lag phase of 10 d, seriously hindering culture performance (Fig. 1B). Therefore, in order to enhance the cell attachment and, consequently, to reduce the lag phase, beads were incubated in FBS prior to cell inoculation (Kim et al., 1992).

Triplicate experiments (*i.e.* cells from three different donors) were carried out under these conditions and using this strategy it was possible to observe that after 24 h, all cells efficiently adhered onto the microcarriers, entering exponential growth phase upon day 1 (Fig. 1B). An average maximum density of 4.2×10^5 cells/mL was obtained after 8 d, which corresponds to a fold increase of 8.4 ± 0.8. The cell viability was constantly higher than 95%, whereas live cells in suspension accounted for <0.5% of total cell number. Specific cell growth rate was slightly higher for the spinner flask culture compared to control (0.41 and 0.36 d^{-1}, with $r^2 = 0.98$ and 0.97, respectively).

Images of MTT and DAPI staining attested cell expansion during the 10 d of culture (Fig. 2A and B). At the end of the culture, the beads were completely populated with cells, which may originate hypoxic niches that can contribute to improve cell proliferation (dos Santos et al., 2010).

Envisaging the clinical application of the expanded cells, the FBS used to pre-coat the beads may be replaced with human plasma or platelet lysate, as demonstrated by others when replacing FBS in culture (Bernardo et al., 2007; Shetty et al., 2007).

2.2.2. Improving feeding scheme to prevent metabolic limitations

In our first experiments focusing the expansion of BM MSC in a microcarrier-based spinner flask system, feeding regime consisted of removing 25% of the exhausted culture medium every 2 d, which was replaced by the same volume of freshly added medium (Andrade, 2007). However, in these studies this feeding scheme resulted in glucose starvation during the exponential growth phase and ammonium concentration reached values close to 3.5 mM, potentially inhibitory to MSC growth (Schop et al., 2009) (Fig. 3). Therefore, we performed a more frequent feeding scheme: 25% medium change everyday and starting only at day 3, in order to prevent the removal of autocrine factors potentially instructive for cell proliferation initiation.

During exponential growth phase, glucose and glutamine concentrations were maintained at a level higher than the respective Monod constants, K_S (about 0.1–0.4 and 0.08–0.15 mM, respectively, for mammalian cells; Acosta et al., 2007; Cruz et al., 1999; Jeong and Wang, 1995) (Fig. 3), suggesting cell growth was not likely limited by the two main nutrients. Lactate and ammonia concentrations were maintained below inhibitory values for human MSC (Schop et al., 2009).

Based on the cell numbers and the consumption/production metabolites, we calculated the average metabolic parameters during the exponential growth phase (Table 1). The apparent yield of lactate from glucose – $Y_{lac/gluc}$ was statistically lower in the spinner flask system (1.88 ± 0.07 and 2.39 ± 0.26, respectively; $p < 0.05$), which indicated that the energy metabolism in spinner culture was significantly more efficient than that in conventional flasks. A value higher than the theoretical maximum (2 mol mol^{-1}) indicates lactate is being produced from other source (*i.e.* glutamine) and metabolic pathways different from glycolysis are being used.

Table 1
Growth and metabolic parameters for the expansion of MSC in *CellSpin* (Integra Biosciences) spinner flasks (50 mL).

μ (d^{-1})	0.41 d^{-1} (r^2 = 0.98)
q_{gluc} (pmol $cell^{-1}$ d^{-1})	−5.4 ± 0.3
q_{lact} (pmol $cell^{-1}$ d^{-1})	10.3 ± 0.9
q_{glut} (pmol $cell^{-1}$ d^{-1})	−0.9 ± 0.1
q_{amm} (pmol $cell^{-1}$ d^{-1})	0.9 ± 0.2
$Y_{lac/gluc}$ (mmol $mmol^{-1}$)	1.88 ± 0.07
$Y_{amm/glut}$ (mmol $mmol^{-1}$)	1.19 ± 0.42

BM MSC (passages 4–6, prepared according to (dos Santos et al., 2010) were used; *Cultispher S* (Sigma) gelatin microcarriers were prepared (Fernandes et al., 2007) and cells (50,000 cells/mL) were seeded on 1–2 g/L of microcarriers (preincubated with FBS (24 h)) and cultured in MesenPRO RS™ under 30 rpm. Average specific growth rate (μ) was calculated from the slope of the graphic representation of $\ln X_v = \ln X_0 + \mu \cdot t$ (Fig. 1B), where X_0 and X_v are the number of viable cells at day 0 and a given time point t, respectively. Specific consumption or production rates were also calculated during the exponential phase (Fernandes et al., 2007). Data represent mean ± SEM.

2.3. Characterization of the expanded cells

Immunophenotypic analysis was performed for cells derived from three different donors (Fig. 4A), and it was observed that CD73 and CD90 were expressed in 98–99% of the cells. On the other hand, CD105 expression was found to be dependent on the time of trypsinization of the microcarriers (Potapova et al., 2008) and ranged 85–95%.

MSC expanded under dynamic conditions were also able to differentiate into osteogenic and adipogenic lineages in response to specific induction media (Fig. 4B).

Finally, the clonogenic capacity of MSC (CFU-F) was assessed before and after the expansion under dynamic conditions (dos Santos et al., 2010). Although the number of colonies with more than 50 cells decreased in 33%, a 25% increase of the number of colonies with cells ranging from 25 to 50 was observed. This slight decrease in colony number normally occurs from a passage to other (DiGirolamo et al., 1999).

3. Conclusions

We present here a comprehensive study focusing the maximization of culture performance by optimization of culture conditions, namely the initial cell seeding and feeding regime, including a complete characterization of the expanded cells in spinner flasks. These results are promising envisaging the use of a fully controlled stirred bioreactor system for the efficient clinical-scale expansion of human MSC for regenerative medicine applications.

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