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Ex Vitro Expansion of Human Placenta-Derived Mesenchymal Stem Cells in Stirred Bioreactor

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Abstract The high demand of human placenta-derived mesenchymal stem cells (hPDMSCs) for therapeutic applications requires reproducible production of large numbers of well-characterized cells under well-controlled conditions. However, no method for fast hPDMSCs proliferation has yet been reported. In the present study, the feasibility of using a stirred bioreactor system to expand hPDMSCs was examined. hPDMSCs were cultured either in stirred bioreactors or in tissue culture flasks (T-flasks) for 5 days. Total cell density and several parameters of physical microenvironments were monitored in the two culture systems every 24 h. The maintenance of the antigenic phenotype of hPDMSCs before and after culturing in the stirred bioreactor system was cytometrically assessed. Data suggested that the physical microenvironment in the stirred bioreactors was much more favorable than that of the T-flasks. At the end of 144 h culturing, the total cell number was increased 1.73 times from the T-flasks to the stirred bioreactors. In addition, hPDMSCs could maintain their antigenic phenotype when cultured in stirred bioreactors. These results provide the initial assessment for large-scale hPDMSCs production using suspension culture bioreactors.

Keywords Mesenchymal stem cells · Stirred bioreactor · Cell expansion · Tissue engineering · Regeneration medicine

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Introduction

Mesenchymal stem cells (MSCs) offer a marrow stromal environment that supports hematopoiesis. These cells possess a wide range of differentiation potentials that enable them to differentiate into bone, adipose, cartilage, muscle, and endothelium when cultured under specific permissive conditions [1, 2]. Moreover, the immunogenicity of MSCs are low and using MSCs from unrelated donors as a cell feeder layer for support of hematopoietic progenitor cells (HPCs) does not induce immunological rejection [3, 4]. Therefore, MSCs have a wide range of potential applications in clinical therapy. It is necessary to develop ex vivo culture systems to scale up the cell proliferation. Conventional culture systems, such as T-flasks and gas-permeable blood bags, are the most widely used devices for expanding MSCs. However, such static culture systems have several inherent limitations. For example, the lack of mixing causes concentration gradients for dissolved nutrient substances and metabolites; the lack of on-line monitoring and controlling of the environmental conditions limits the productivity of cell numbers. Stirred bioreactors, on the other hand, can provide balanced nutrition for MSCs expansion with controlled optimal environmental conditions. Placenta, a biological waste after delivery, along with the ease of its accessibility and lack of ethical concerns, has become an attractive source of mesenchymal stem cells for basic and clinical applications. In this work, the feasibility of using stirred bioreactors to expand human placenta-derived mesenchymal stem cells (hPDMSCs) was studied. Parallel experiments using T-flasks were also carried out for a comparative study. During the 6 days of culturing, total cell density, antigenic phenotype, cell metabolites, medium pH, and osmolality were monitored and compared between the two methods.

Materials and Methods

Isolation and Culturing of hPDMSCs

Term (38–40 weeks gestation, $n=10$) placentas from healthy donor mothers were obtained with informed consent approval according to procedures of the institutional review board. The deciduas were stripped and dissected into small pieces. The harvested pieces of placenta were then washed several times with phosphate-buffered saline (PBS) and mechanically minced. The minced pieces were subsequently laid uniformly on petri dishes and cultured in Dulbecco′s modified Eagle′s medium low glucose (DMEM) (Invitrogen, GIBCO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, USA), 0.1 mM β-mercaptoethanol (Sigma, USA), 2 mM L-glutamine (Invitrogen, USA), 0.1 mM non-essential amino acids (MEM) (Invitrogen, USA), maintained at 37 °C in a humidified atmosphere with 5% CO_2. Medium was replaced every 3 days. When cells were over 80% confluent, they were recovered with 0.25% trypsin and replated at a dilution of 1:3.

hPDMSCs Culture Preparations for the Stirred Bioreactors

Microcarriers were first soaked at a concentration of 10 mg/ml Cytodex™-3 (diameter141-211 μm, GE Healthcare Bio-Sciences AB, Sweden)in PBS overnight. The next day, PBS was discarded and the microcarriers were sterilized at 121 °C for 25 min. They were then washed with 10% DMEM once and soaked in 10% DMEM at 4 °C until use. The stirred

bioreactors were brushed and rinsed three times with deionized water before soaked in 75% ethanol overnight. The next day, reactors were again washed three times with deionized water. Subsequently, the stirred bioreactors were wrapped in aluminum foil separately and autoclave-sterilized at 121 °C for 25 min. Afterwards, they were dried in the oven.

The hPDMSCs at the third passage were inoculated to the microcarriers with known density to prepare the cell suspension. The cell suspension was incubated at 37 °C in a humidified atmosphere for 24 h before it was injected into the stirred bioreactors. The final density was 1×10^5 cells/ml. Stirring speed of the reactor was step increased from 40 rpm for 6 h to 60 rpm. hPDMSCs were also cultured in T-flasks at a final density of 1×10^5 cells/ml. Both the bioreactors and T-flasks were maintained at 37 °C and 5% CO_2 in a humidified atmosphere.

Monitoring of Micro-Environmental Parameters

Cell suspension was sampled every 24 h. About 2 ml cell medium was drawn out from the stirred bioreactor and the T-flask respectively each time. Meanwhile, the same amount of fresh medium was added back to the reactor and the T-flask. pH and osmolality of the samples were immediately measured with a Thermo Orion Benchtop pH meter (550 A, KY, USA) and a vapor pressure osmometer (Model 5520, Wescor, Inc., UT, USA). The sample media was then kept at −20°C for glucose (GL) measurement by glucose oxidase method and lactic acid (LA) analysis by gas chromatography following standard protocols.

Cell numbers were monitored every 24 h using a hemocytometer and trypan blue dead cell exclusion method. The experiment was terminated when cells numbers reached stationary phase. All cells were harvested and stained for 30 min at 4 °C with the following PE- or FITC-conjugated antibodies: anti-CD13-PE, anti-CD14-PE, anti-CD29-PE, anti-CD73-PE, anti-CD90-PE, anti-CD166-PE, anti-HLA-DR-PE, anti-CD31-FITC, anti-CD44-FITC, anti-CD45-FITC, anti-CD105-FITC, and anti-HLA-ABC-FITC. Stained cells were washed with PBS and analyzed with a flow cytometer following standard protocol.

Statistical Analysis

All experiments were repeated three times. Results are expressed as mean±standard error of the mean (SEM). Mean values of different populations were compared by analysis of variance. Rate of different populations were compared by χ^2-test using software SPSS version 10.0. $P<0.05$ was considered statistically significant.

Results

Characterization of Placenta-Derived Cells

Three days after culturing, small amount of fibroblast-like cells started to appear around the placental specimens. Colonies formation started after approximately 9–14 days and cell culture became over 80% confluent after 2 weeks (Fig. 1a). After trypsinization, the placenta-derived cells could form colonies again (Fig. 1b) and could proliferate more than 20 passages.

The antigenic phenotype of all cell preparations were examined carefully by flow cytometry. Immunophenotyping of hPDMSCs revealed these cells were positive for many markers common to BMSCs, such as CD13, CD29, CD44, CD73, CD90, CD105, CD166,

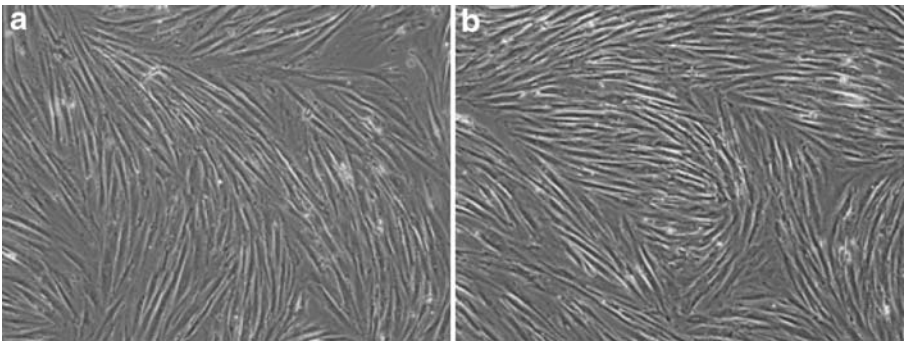

Fig. 1 Morphology of placenta-derived mesenchymal stem cells (hPDMSCs) (×200 magnification). Three to 14 days after inoculation, fibroblast-like cells were transferred from the placenta specimens. Cells formed individual colonies as showed in (**a**). After the third passage, hPDMSCs presented the morphology of fibroblast-like cells, as shown in (**b**)

and HLA-ABC. Meanwhile, hPDMSCs were negative for the hematopoietic surface markers of CD14, CD31, CD45, and HLA-DR (Fig. 2).

No significant difference was observed in the expression of cell surface proteins of CD13, CD14, CD29, CD31, CD44, CD45, CD73, CD90, CD105, CD166, HLA-ABC, and HLA-DR before and after bioreactor culturing in the two culturing systems ($P>0.05$) (Fig. 3).

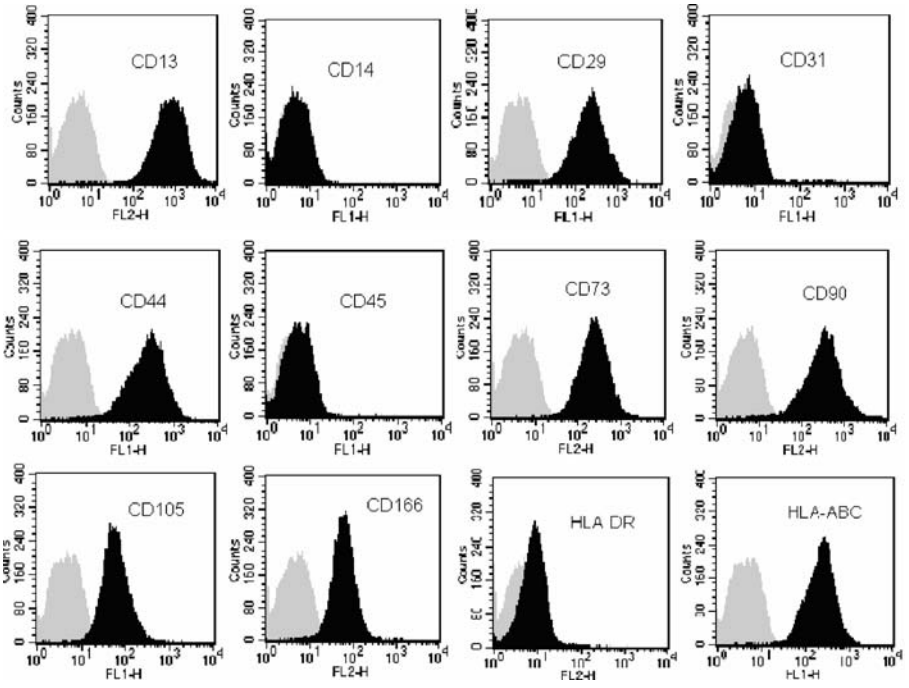

Fig. 2 Phenotype of hPDMSCs grown in the stirred bioreactor. Flow cytometry analysis demonstrated that the hPDMSCs were positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC, but negative for CD14, CD31, CD45, and HLA DR

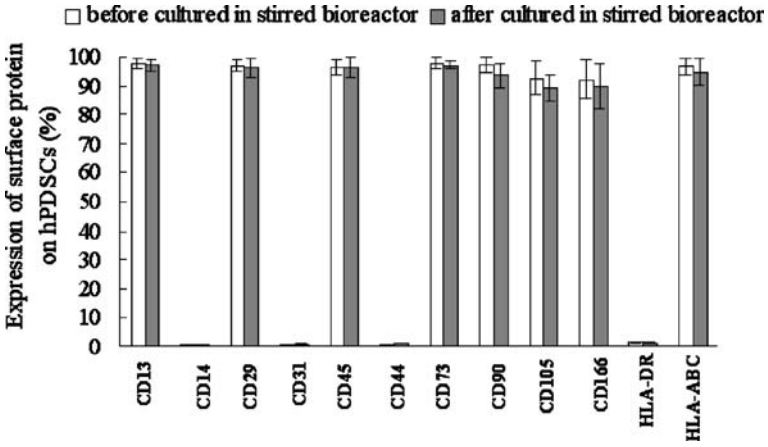

Fig. 3 Flow cytometry analysis of phenotype change of hPDMSCs before and after culturing in stirred bioreactors

Observations of hPDMSCs Growth on Microcarriers

The growth characteristics of hPDMSCs on microcarriers were observed throughout the course of experiment. The hPDMSCs proliferated much slower in the T-flasks. After

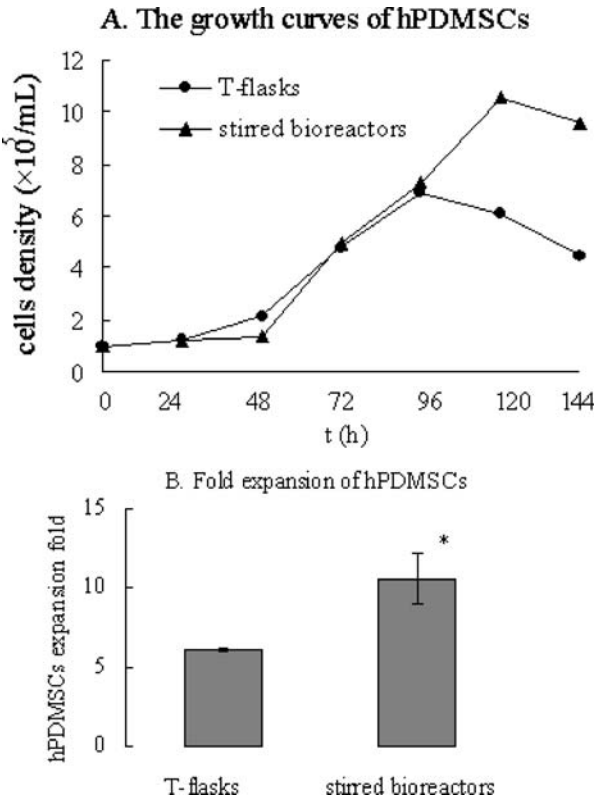

Fig. 4 Comparison of expansion rate of hPDMSCs on microcarriers between stirred bioreactors and T-flasks. hPDMSCs cells were seeded at a density of 1×10^5 cells/ml. **a** The growth curves of hPDMSCs. **b** Fold expansion of hPDMSCs (at 120 h). *Asterisk* indicate $P<0.05$

4 days, a plateau in the growth was reached. Thereafter, the total cell density decreased due to the detachment of some cells from the microcarriers when cells reached growth inhibition phase. In the stirred bioreactors, a smaller fraction of cells fell down from the microcarriers. Some cells died because of the shearing force from the stirring at the first 48 h. Over all, cells proliferated more rapidly than those in the T-flasks (Fig. 4a). Figure 4b summarized hPDMSCs expansions in the stirred bioreactor vs. T-flasks. The average expansion fold increase in stirred tank bioreactors was 10.55±1.62 significantly higher than that in the T-flasks of 6.10±0.11 ($P<0.05$).

Microenvironment Changes and Biological Assays

The average concentration of glucose in stirred bioreactors was higher than that in the T-flasks from 24 h to 144 h time points, but the difference was not statistically significant ($P>0.05$). While the average concentration of lactic acid was continuously lower than that in the T-flasks from 48 h to 144 h, and only the differences at 96, 120, and 144-h time points were statistically significant ($P<0.05$) (Fig. 5). This may be due to the well mixing of the

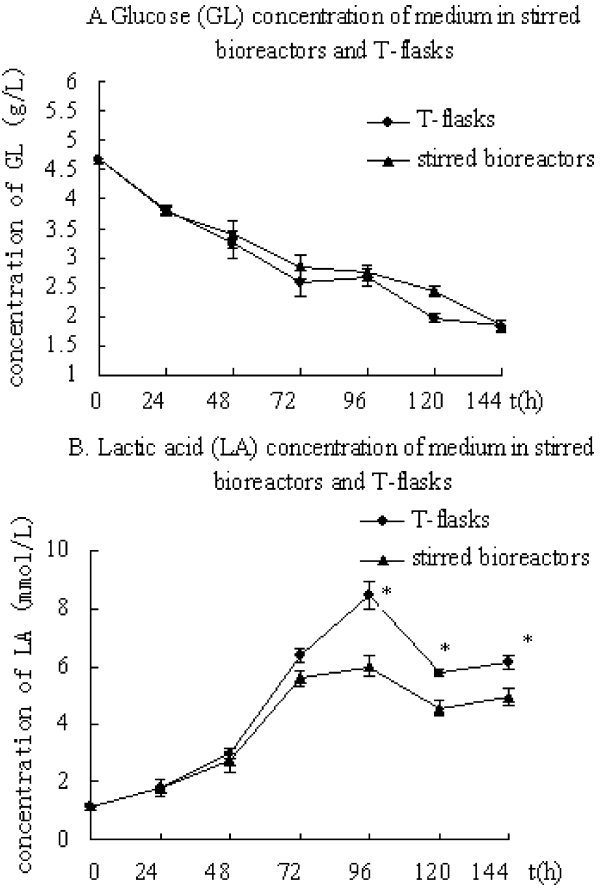

Fig. 5 Change of glucose (GL) and lactic acid (LA) levels of the medium during hPDMSCs culturing in stirred bioreactors and T-flasks: **a** GL; **b** LA. *Asterisk* indicate $P<0.05$

nutrient substances by the action of stirring. Majority of the energy for cell growth was provided by Krebs cycle pathway.

The regulation of hPDMSCs was not only controlled by the cell microenvironment, but also by local pH and osmolality. As seen in the Fig. 6, pH was mostly kept between 6.8 and 7.4 in the stirred tank bioreactors, while it was 6.5 to 7.4 in the T-flasks. Osmolality of the culture medium in the stirred tank bioreactors and T-flasks were very similar, both were maintained between 300 and 320 mm Osmol/kg throughout the culturing period ($P>0.05$).

We also analyzed the viability of hPDMSCs. Which were induced to differentiate into chondroblast and osteoblasts by 10 ng/ml TGF-ß, 100 ng/ml insulin, 10^{-7} mol/L decaesadril, 6.25 μg/ml siderophilin, 10 mmol/L ß-sodium glycerophosphate, 50 μg/ml antiscorbic acid; cardiomyocytes with "hanging drop" methods. Chondroblast and osteoblasts were observed with Toluidine Blue and von-Kossa staining. Cardiomyocyte genes *α-MHC*, *ANF*, and *MLC-2v* expression were analyzed by RT-PCR. Before and after cultured in stirred bioreactors, hPDMSCs could be differentiated into chondroblast, osteoblasts, and cardiomyocytes in vitro (data not shown).

Overall, results indicated that the stirred tank bioreactors were more suitable for the expansion of hPDMSCs.

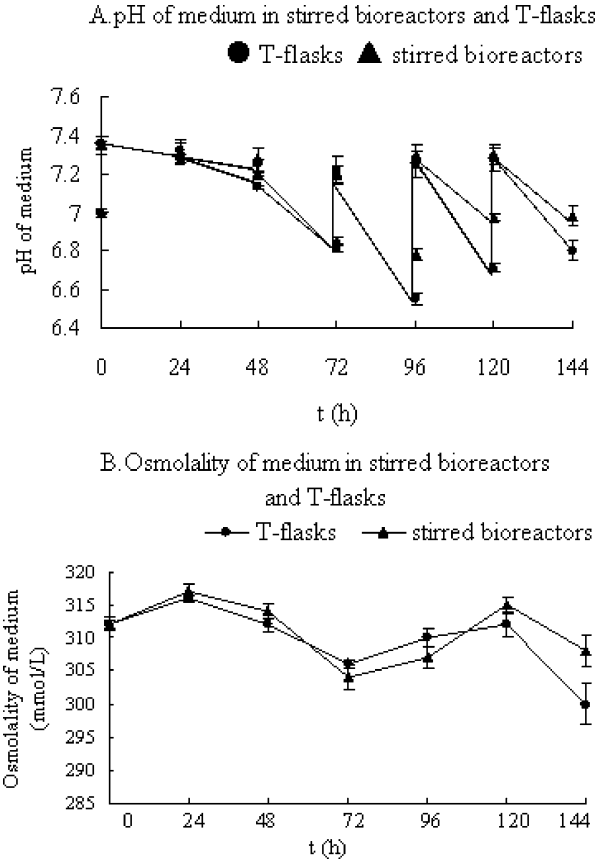

Fig. 6 Change of pH and osmolality of the medium in the stirred bioreactors and T-flasks during hPDMSCs culturing: **a** pH; **b** osmolality

Discussion

Due to the easy accessibility, lack of ethical concerns, and abundant cell numbers, hPDMSCs have become an attractive alternative source of progenitor cells for basic research [1–4]. Conventional culture systems such as T-flasks and gas-permeable blood bags are the most widely used devices for expanding hPDMSCs. However, with the rapid development of biotechnology, conventional culture systems could not keep up with the growing demands. Moreover, such static culture systems have several inherent limitations, such as lack of mixing resulting in concentration gradients of dissolved nutrient substances and metabolites, and lack of readily online monitoring and control of the environmental conditions which greatly limits the cell expansion [5]. Therefore, a large scale hPDMSCs culture was tested using bioreactors in this study. Bioreactors can provide balanced nutrition for cells expansion and the environmental conditions can be easily monitored [6]. In fact, in many other aspects, bioreactor technology for large-scale cell culturing has been implemented and the standard stirred-tank reactor is deemed as the technology of choice [7].

The stirred bioreactor, powered by a motor for mixing the culture, can provide balanced and homogenous nutrition in the 3D culture environment, allowing cells to communicate, and contact each other [8]. The stirred bioreactor system can be used for both suspension cells and adherent cells. When microcarriers or porous micro spheres are used to culture adherent cells, the final cell density could reach about 10^7 cells/ml [9]. Chen et al. expanded MSCs (derived from bone marrow) in a rotary bioreactor. After 8 days of bioreactor treatment, the average net expansion of MSCs was approximately ninefold and the expanded MSCs maintained their stemness [10]. In this study, hPDMSCs proliferation using microcarriers in stirred bioreactors was found to be significantly more effective than T-flask. More hPDMSCs were attached to microcarriers in the bioreactors. When hPDMSCs reached a growth plateau at the fifth day, the average expansion fold in stirred tank bioreactors was 10.55±1.62 per passage, significantly higher than that in the T-flasks (6.10±0.11, $P<0.05$). Meanwhile, hPDMSCs in stirred bioreactor were able to maintain their phenotype as in the T-flasks. The investigation on the relation of stress and cell growth revealed that cells growth was certainly affected by mechanical stresses [11–14]. The stimulus of fluid mechanics was suggested to increase cell proliferation and induce synthesis of DNA [15]. Consistent with those reports, data from this study demonstrate that stirred bioreactors could provide not only optimized culturing conditions for cell growth, but befitting mechanical environment to enhance cell proliferation.

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