Expansion of adipose derived mesenchymal stem cells using shake flasks

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Abstract
The expansion of stem cells, including mesenchymal stem cells (MSCs), has been successfully demonstrated using microcarrier-based small bioreactors such as spinner flasks. In this study, we explored a simple alternative for microcarrier-based MSC expansion using conventional shake flasks. This method relies on a new type of shake with built-in CO₂ gas control capability (incubator shake). The expansion of adipose-derived mesenchymal stem cells (AdMSCs) was compared between shake flasks and spinner flasks using microcarriers. The AdMSCs were seeded at a density of 3 × 10⁴ cells/cm² in either a baffled shake flask or a spinner flask, each containing 0.5 g plastic microcarriers (cross-linked polyethylene) and 40 ml of stem cell growth medium (serum and growth supplement-free). For the initial attachment of cells, the agitation speed of the incubator shaker and rotation speed of the spinner (housed inside of a CO₂ incubator) were set at 50 rpm and both systems were set to maintain CO₂ at 5%. After 2 hours of incubation, the cell culture volume was adjusted to 50 ml of total volume with 10 ± ml of medium containing serum 4% (final concentration of FBS) and growth supplement (10 μg/ml final concentration of rhFGF basic, rh FGF acidic, rh EGF and 4.8 mM final concentration of L-Alanyl-L-Glutamine). Following the addition of FBS and growth supplements, the impeller speed of the spinner flask and rotation of the incubator shaker were set at 70 rpm. Cells attached to the microcarrier beads and entered into the stationary phase of growth within 3 days of culture. During early growth phase (day 2), AdMSCs had 2.44-fold higher cell numbers under shake flask culture as compared to spinner flask culture. Another experiment was performed under both shake and spinner culture conditions where cells were inoculated at 10x lower density (3 × 10³ cells/cm²) in order to avoid overcrowopulation and to retain the differentiation properties of stem cells. This experiment was performed for 12 days and each day samples were collected for cell number and metabolite measurements. Cell number measurements revealed that AdMSCs grown under shake culture conditions had 1.8-fold and 1.6-fold higher cell numbers than spinner flasks during early log (day 3 and stationary (day 9) phases of growth. Extracellular metabolite measurements revealed that glucose concentrations decreased from 1.00 g/l to 0.548 g/l (for shake flasks) and 0.798 g/l (for spinner flasks), whereas lactate concentrations increased from 0.042 g/l to 0.396 g/l (for shake flasks) and 0.259 g/l (for spinner flasks) after 12 days of cultures growth. Further, during early growth phase (day 3), the ammonium concentration in spinner flask culture was 1.8-fold higher as compared to shake flask culture. Finally, the growth of MSCs under shake flask conditions did not alter their stem cell properties, which was evident by their ability to differentiate into adipocytes and osteocytes.

Introduction
Stem cells are undifferentiated cells which have the capability of self-renewal and the potential to divide for a long period of time. They have the ability to differentiate into various specialized cells when appropriate growth factors and conditions are provided. Stem cells can be broadly classified as: embryonic, adult and induced pluripotent stem cells (iPSCs). Adult stem cells can be further characterized by their tissue of origin, such as hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicle stem cells. Recently, most of the studies performed on adult stem cells are either hematopoietic or adipose-derived mesenchymal stem cells. Like any other adult stem cells, adipose-derived mesenchymal stem cells (AdMSCs) express all of the common stem cell markers and can be differentiated into various types of specialized cells under appropriate growth conditions. AdMSCs have advantages over other mesenchymal stem cells (MSCs), since they can be isolated in large quantities from fat tissue and are resistant to apoptosis.

Although MSCs have enormous advantages for regenerative medicine, drug screening and drug discovery, their applications are limited by the quantities required for industrial or clinical applications². In this study, we developed a simple shake flask culture technique to expand MSCs on microcarrier beads, which can be used to scale-up into large scale bioreactors.

Methods
Cell culture
AdMSCs were obtained from ATCC®(PCS-500-011) at passage 2 and cells were seeded at a density of 5,000 cells/cm² to expanded the cells in a T-75 cm² flask.

Cultivation of cells on microcarriers
In order to attach the AdMSCs to microcarrier beads (Solsorb®), the cells were initially seeded at a density of 3 × 10³ and 3 × 10⁴ cells/cm² in either a pre-siliconized baffled shake flask or a spinner flask, each containing 40 ml of basal mesenchymal stem cell medium. For the initial attachment of cells, the agitation speed of the Eppendorf New Brunswick™ 5411 incubator shaker and rotation speed of the spinner (housed inside of a Galaxy® 170 CO₂ incubator) was set at 50 rpm and incubated for 2 hours at 37°C with 5% CO₂. After 2 hours, the cell culture volume was adjusted to 50 ml of 50 ml total volume with 10 ml of medium containing serum 4% (final concentration of FBS) and growth supplements (10 μg/ml final concentration of rhFGF basic, rh FGF acidic, rh EGF and 4.8 mM final concentration of L-Alanyl-L-Glutamine). Following the addition of FBS and growth supplements, the impeller speed of the spinner flask and rotation speed of the 5411 incubator shaker were set at 70 rpm. After 18 to 24 hours incubation, 1 ml of samples (microcarriers and media) was collected for microscopic observations and to determine the attachments of cells to microcarriers.

Cell counting
Cells on microcarrier beads were counted by hemocytometer.

Metabolite measurement
The supernatants collected during cell counting were used for metabolite measurement using the automated YSI® 2950 bio-analyzer.

Stem cells surface markers identification assay
Identification of stem cell markers was done by immunostaining using anti-human CD44 and CD90 antibodies (BioLegend®) and visualized under EVOS® FL microscope.

Differentiation assays
AdMSCs were harvested from both shake and spinner flasks into 50.0 ml tubes. Adipocytes and osteocytes differentiations were performed on microcarrier expanded cells using differentiation assay kits from ATCC®. Adipocyte and osteocyte differentiated cells were identified by staining with either Oli red O or Alizarin red S kits (ScienCell) according to manufacturer instructions and visualized under OLYMPUS® CKX40 microscope.

Results

Conclusions
> Stem cell expansion using shake flask conditions appears to be a viable and simple alternative to the spinner flask system.
> Our newly developed shake flask culture conditions reduce shearing, eliminate potential cell damage by the spinner rod, decrease the risk of contamination associated with using a magnetic stirrer base into the CO₂ incubator and as such, reduces experimental complexity.
> This method also greatly increases the cell culture capacity whereby multiple shake flasks can be placed in the incubator shaker simultaneously.
> In the case of spinner flask culture, a typical incubator without active cooling can only handle the heat emitted from a single magnetic stirrer base before causing temperature set-point overshoot, a significant limitation to the scale-up potential of the spinner method. This reinforces the superiority of using shake flasks in the incubator shaker for increased capacity.