APPLICATION NOTE No. 259 I September 2013

A Novel Method for the Expansion of Mesenchymal Stem Cells using a New Brunswick™ S41i CO_2 Incubator Shaker

Khandaker Siddiquee and Ma Sha, Eppendorf Inc., Enfield, CT, U.S.A

Abstract

The expansion of stem cells, including mesenchymal stem cells (MSC), 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 CO_2 incubator with built-in shaking capability, i.e. New Brunswick S41i CO_2 Incubator Shaker. 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 $3x10^3$ cells/cm^2 in both setups, each containing 0.5 g plastic microcarriers and 40 mL of stem cell growth medium.

The cell culture experiments were conducted for 12 days and samples were collected daily for analysis of cell growth, biochemistry and metabolites. Cell density studies revealed that AdMSCs cultured under shake flask conditions achieved excellent growth under 12 day batch-culture conditions.

Lastly, the AdMSCs expanded using the shake flask method remained high quality stem cells, which was evident by CD44 and CD90 stem cell marker assays and their ability to differentiate into either adipocytes or 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 (iPS). 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. Most of the studies performed on adult stem cells utilize either hematopoietic or adipose-derived mesenchymal stem cells[1]. Like 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[2].

Although MSCs have enormous advantages for regenerative medicine, drug screening and drug discovery, their applications are limited by the quantities required for industrial applications[3]. 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. The microcarrier shake flask culture, which requires both agitation and CO_2 gas control, was conducted in the Eppendorf New Brunswick S41i CO_2 incubator shaker.

The New Brunswick S41i CO_2 Incubator Shaker, designed for both non-adherent and adherent cell culture applications, combines the precise temperature and CO_2 control of an incubator with the reliable New Brunswick laboratory shaker drive mechanism. Key features include sealed inner/outer doors, high-temperature disinfection, and reduced CO_2 consumption compared to competitor models[4].

Materials and Methods

Initial cell culture in T-Flasks
AdMSCs were obtained from ATCC® (PCS-500-011) at passage 2 and cells were seeded at a density of 5,000 cells/cm^2 into a T-75 cm^2 flask (Eppendorf) using 15 mL of mesenchymal stem cell basal medium (ATCC) supplemented with 2 % fetal bovine serum, 5ng/mL rh FGF basic, 5ng/mL rh acidic, 5 ng/mL rh EGF and 2.4 mM L-Alanyl-L- Glutamine (ATCC).

Cultivation of cells on microcarriers
Prior to start of the experiment, 0.5 g of 125-212 micron polystyrene microcarriers (SoloHill®) (180 cm^2 for a 50 mL culture) was transferred into a siliconized (Sigmacoat®; Sigma) 250 mL spinner flask (Corning®) and shake flasks (Schott®, Duran®) along with 25-30 mL of PBS. The flasks were then autoclaved at 121 °C for at least 30 minutes. Microcarriers were allowed to settle to the bottom of the shake/spinner flasks and the autoclaved PBS buffers were carefully aspirated with the electronic pipetting aid easypet® (Eppendorf) equipped with a 25 or 50 mL pipette. The AdMSCs were initially seeded at a density of 3x10^3 cells/cm^2 into both flasks, each containing 40 mL of basal mesenchymal stem cell medium. For the initial attachment of cells, the agitation speed of the New Brunswick S41i CO_2 incubator shaker and rotation speed of the spinner (housed inside of an Eppendorf Galaxy® 170 R CO_2 incubator) were both kept at 50 rpm and incubated for 2 hrs at 37 °C with 5 % CO_2. After incubation, the cell culture volume was adjusted to 50 mL total with 10 mL of medium containing serum to reach a final FBS concentration of 4 % and targeted final concentration of growth supplements (10 ng/mL final concentration of rh FGF 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 rotation speed of the spinner and the agitation speed of New Brunswick S41i CO_2 incubator shaker were both raised to 70 rpm. After 18 to 24 hrs of incubation, 1 mL of homogeneous samples containing both media and microcarriers were collected for microscopic observations, cell counting as well as biochemistry analysis.

Cell counting
Cells on microcarrier beads were counted by hemocytometer. To accomplish this, microcarrier beads were suspended in citric acid solution containing crystal violet (0.1 % crystal violet in 0.1 M citric acid solution) equal to the volume of supernatant removed from the tube. The contents of the tube were incubated for 1 hr or overnight at 37 °C and vortexed for a few seconds to release the stained nuclei. The nuclei were counted with hemocytometer.

Biochemistry and metabolites analysis
The supernatants collected during cell counting were used for biochemistry and metabolite measurements using an automated YSI® 2950 Bio-analyzer.

Stem cell surface marker assay
To assess the quality of AdMSCs after expansion and to confirm that the stem cell markers were retained during the microcarrier-based culture, CD44 and CD90-specific fluorescent immunoassays were performed using the following procedure. 5 mL samples were collected from both the spinner and shake flasks near the end of microcarrier culture. After the microcarriers settled to the bottom, the supernatants were removed and the microcarrier beads containing cells were gently washed 3 times with PBS at room temperature. Cells on the microcarrier beads were then fixed with 4 % paraformaldehyde for 30 minutes followed again by PBS washing 3 times. Cell-containing microcarrier beads were blocked with 5 % FBS at room temperature for 1 hr and immunostained with FITC-conjugated antihuman CD44 (BioLegend®) and APC-conjugated antihuman CD90 (BioLegend®) antibody solutions, also for 1 hr at room temperature. The beads were washed 5 times with room temperature PBS for 5 min and visualized using an EVOS® FL fluorescence microscope.

Stem cell differentiation assays
AdMSCs were harvested from both shake and spinner flasks into 50 mL tubes. Once the microcarrier beads settled to the bottom of the tube, the supernatants were removed and cells were washed with DPBS. Afterwards, the microcarrier beads were treated with 5 mL of prewarmed trypsin-EDTA solution at 37 °C for 10 min. During incubation, the tubes were occasionally vortexed for 2 sec and then neutralized by adding an equal volume of trypsin neutralizing solution. Microcarrier beads were a llowed to settle to the bottom of the tube and the supernatants were collected as soon as possible. Microcarrier beads were washed 2-3 times with DPBS and as much supernatant as possible was collected into a 50 mL tube. Following washing, AdMSCs were collected to bottom of the tube by centrifugation at 120 xg for 5 min and resuspended in 5 mL of mesenchymal stem cell medium. Cells were seed at a density of 18,000 cells/cm^2 into a 24-well plate. Adipocytes and osteocyte differentiations were performed on those cells using differentiation assay kits from ATCC. Adipocyte and osteocyte differentiated cells were identified by cell-type specific staining with either Oil red O or Alizarin red S kits (ScienCell®) according to manufacturer instructions and visualized using an OLYMPUS® CK40 microscope.

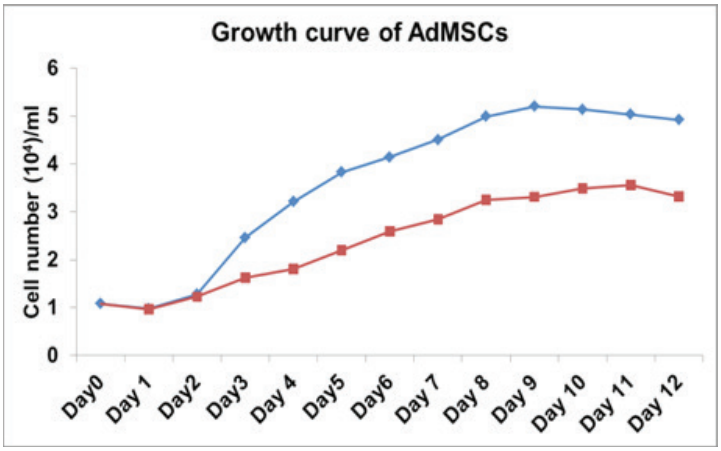
Results and Discussion

To compare between shake flask and spinner flask cultures, AdMSCs were seeded at a density of 3x10^3 cells/cm^2 in both systems. Cell culture studiess were conducted for 12 days and samples were collected for cell growth, biochemistry and metabolite analysis daily. Cell growth studies revealed that AdMSCs cultured under shake flask conditions achieved excellent growth during the 12 day batch culture (Figure 1A). Biochemistry and metabolite analysis revealed that glucose concentrations decreased from 1.09 g/L to 0.548 g/L (for shake flask culture) and 0.798 g/L (for spinner culture), whereas lactate concentrations increased from 0.042 g/L to 0.396 g/L (for shake flask culture) and 0.259g/L (for spinner culture) after 12 days of culture (Figure 1B &C).

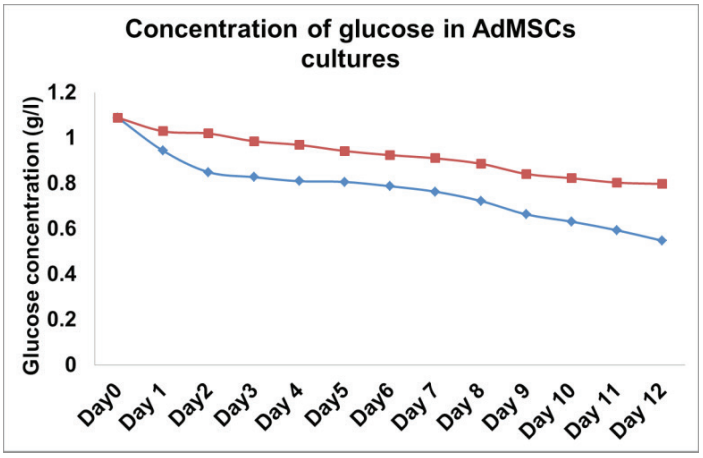
The higher glucose consumption and lactate production rate seen in the shake flask culture supports the finding that the stem cells grew at a faster rate under the shake flask conditions. Furthermore, during early growth phase (day 4); the amount of ammonium accumulated in spinner flask culture (2.4 mM) was 1.8-fold higher than shake flask culture (1.3 mM) (Figure 1D). It has been shown that even low level of ammonium (1.9 mM) inhibits MSC growth[5]. The spinner culture has shown ammonium level exceeding 2 mM early and throughout the culture process, which indicates the slower growth by the spinner method could be a result of ammonium toxicity-induced growth inhibition. The fact that spinner culture had elevated ammonium levels early in the culture not seen in the shake flask also indicates possible stem cell damage due to shear force by the spinner rod. The spinner rod was observed to display a “stop & go” motion at low speeds; precise speed control is not possible, especially at low rotation speeds. However, our observations were based on the specific spinner device available at our research facility, the results may not represent typical or average performance from spinner devices available in the market place.

To determine whether or not AdMSCs retained their stem cell properties during their growth under shake flask conditions, immunostaining of stem cell surface markers and differentiation assays were performed. Microcarrier beads that contained AdMSCs were immunostained with stem cell surface marker antibodies such as: FITC-conjugated antihuman CD44 and APC-conjugated antihuman CD90 and revealed that AdMSCs retained stem cell surface markers during growth under shake flask culture condition (Figure 2A&B). For the adipocyte and osteocyte differentiation assays, AdMSCs were collected from the microcarrier beads and seeded into 24 well plates that contained either adipocyte or osteocyte differentiation medium. After 17 days of culture, the plates were stained with Oil Red O or Alizarin Red S staining solutions, respectively. Microscopic observation indicated that most of the AdMSCs from shake flask culture differentiated into either adipocytes or osteocytes successfully (Figure 3A&B).

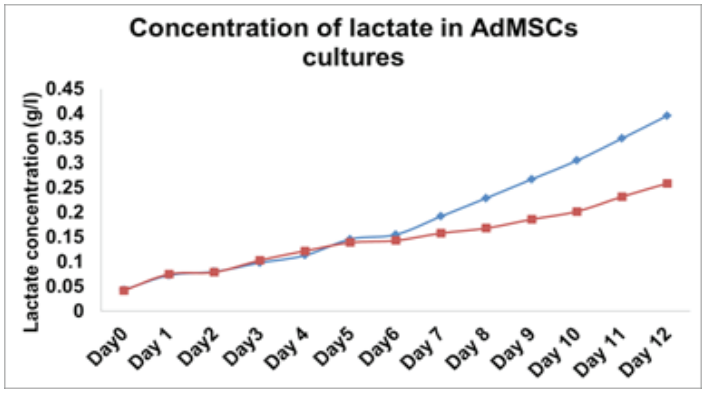
A.



B.



C.



D.

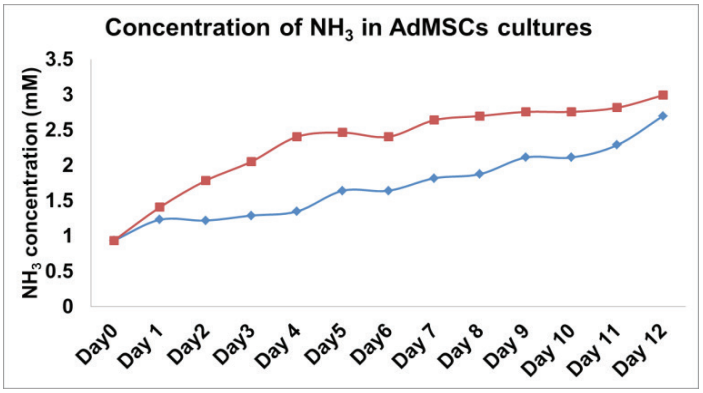
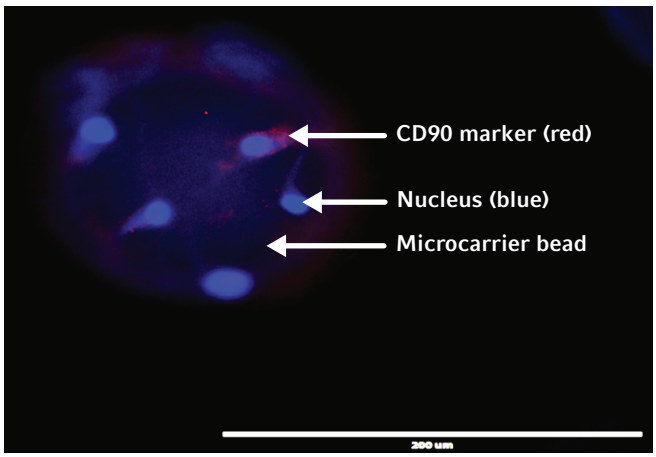


Figure 1. Analysis of AdMSCs growth and metabolism in shake flask and spinner flasks culture conditions: A) growth; B) glucose utilization; C) lactate production and D) ammonium production.(◆) shake flask and (■) spinner flask.

A.



B.

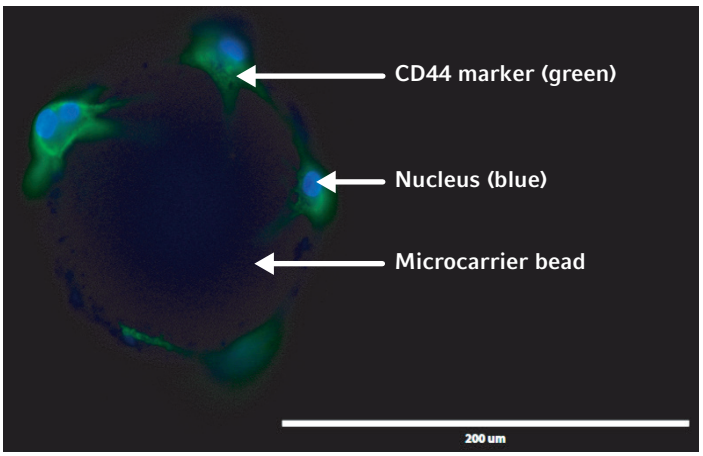
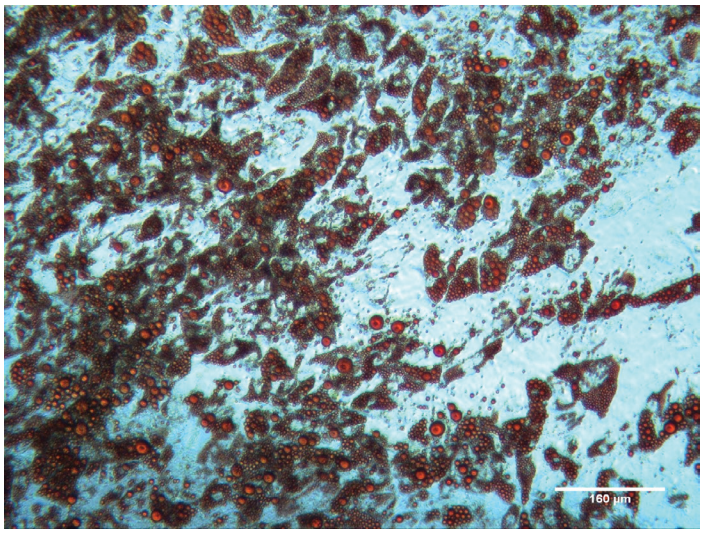


Figure 2. Stem cell marker identification assay for AdMSCs expanded on microcarriers in shake flask. A) AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging. B) AdMSCs on microcarrier beads are positive for CD 44 stem cell marker, as indicated in green by Fluorescence Imaging. Blue color indicates stem cell nuclear staining by DAPI.

A.



B.

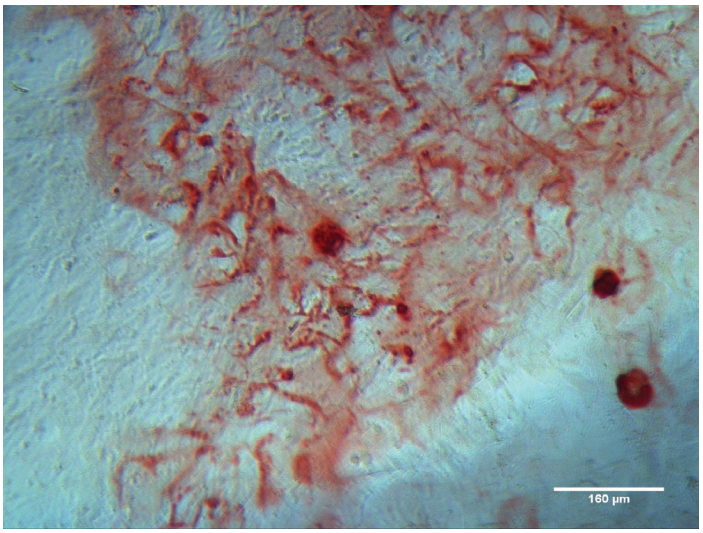


Figure 3. Differentiation assays for AdMSCs expanded on microcarriers in shake flask. A) Adipogenic differentiation formed lipid droplets as indicated by Oil red O positive staining. B) Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by Alizarin Red S positive staining.

Conclusions

Stem cell expansion using shake flask conditions appears to be a viable and simple alternative to the spinner flask system. This novel method relies on a new type of CO_2 incubator with built-in shaking capability, such as the New Brunswick S41i CO_2 Incubator Shaker. The New Brunswick S41i reduces shearing, eliminates potential cell damage by the spinner rod, decrease the risk of contamination associated with inserting a magnetic stirrer base into the CO_2 incubator and reduces experimental complexity. This method also greatly increases the cell culture capacity whereby a large number of shake flasks can be placed in the New Brunswick S41i simultaneously. In the case of spinner flask culture, a typical incubator without active cooling can only handle the heat emitted from a very limited number of magnetic stirrer bases before causing temperature setpoint overshoot, a significant limitation to the scale-up potential of the spinner method. This reinforces the superiority of New Brunswick S41i CO_2 Incubator Shaker as an alternative to incubator/spinner based stem cell culture. This method eliminates a scale-up bottleneck while providing the highest quality stem cell culture for inoculation of large scale industrial bioreactors. The shake flask has lower cost and less parts to disassemble, clean, assemble and autoclave.

References

1. Zhang et al. "Mechanisms Underlying the Osteo- and Adipo-Differentiation of Human Mesenchymal Stem Cells". The Scientific World Journal (2012).Volume 2012; Article ID793823;

2. Nastran el al. "Osteogenic Differentiation and Osteochondral Tissue Engineering Using Human Adipose-Derived Stem Cells." Biotechnology progress (2013) Volume 29.No.1: 176- 185;

3. Schop et al. "Expansion of human mesenchymal stromal cells on microcarriers: growth and metabolism" J. Tissue Eng Regen Med (2010); Volume 4. No. 4; 131-140.

4. Kohlstrom et al. "Hybridoma and CHO Cell Culture using New Brunswick S41i – An Environmental Friendly, Low Emission Incubator Shaker". Eppendorf Application Note AA257, Nov. 2012.

5. Schop D. "Growth and Metabolism of Mesenchymal Stem Cells Cultivated on Microcarriers", Ph.D. Thesis 2010, University of Twente, The Netherlands.

Ordering information

Description	International order no.	North America Order no.
New Brunswick™ S41i CO_2 Incubator Shaker, 120 V, 60 Hz		S41I-120-0100
New Brunswick™ S41i CO_2 Incubator Shaker, 230 V, 50 Hz	S41I-230-0100	
Options		
Stacking kit	P0628-6502	P0628-6502
Additional perforated shelf	P0628-6181	P0628-6181
Interchangeable platforms, 612 x 356 mm (24 x 14 in)	M1334-9920	M1334-9920
Universal platform	M1334-9921	M1334-9921
250 mL Dedicated platform	M1334-9922	M1334-9922
500 mL Dedicated platform	M1334-9923	M1334-9923
1 L Dedicated platform	M1334-9924	M1334-9924
2 L Dedicated platform	M1334-9925	M1334-9925
2.8 L Dedicated platform	M1334-9926	M1334-9926
4 L Dedicated platform	M1334-9927	M1334-9927
Galaxy 170 R CO_2 Incubator (High-Temp Disinfection,1-19 % O2 Control)	CO170R-230-1200	CO170R-120-1200
T-75 Tissue Culture Flask 5 flasks per bag, 100 flasks per case (Available in China, India and Italy)	0030 711.106	
Easypet® (Electronic pipette controller– suitable for serologiocal pipettes, 0.1 to 100 mL)	4421 000.013	022230204

Your local distributor: www.eppendorf.com/contact
Eppendorf AG • 22331 Hamburg • Germany
eppendorf@eppendorf.com

www.eppendorf.com

SoloHill® is a registered trademark of SoloHill Engineering, Inc., Michigan, USA. Sigmacoat® is a registered trademark of Sigma-Aldrich, USA. Duran® is a registered trademark of DURAN Group, GmbH. Corning® is a registered trademark of Corning, Inc., New York, USA. ATCC® is a registered trademark of American Type Culture Collection, Corp., Virginia, USA. YSI® is a registered trademark of YSI, Inc., Yellow Springs, USA. EVOS® is a registered trademark of Life Technologies, New York, USA. BioLegend® is a registered trademark of BioLegend, Inc., California, USA. ScienCell® is a registered trademark of ScienCell Research Laboratories, California, USA. OLYMPUS® is a registered trademark of Olympus Corporation, USA. NucleoCounter® is registered trademark of ChemoMetec, Denmark. Galaxy® is a registered trademark of New Brunswick Scientific, Co., Inc, USA. Eppendorf® and the Eppendorf logo, and Easypet® are registered trademarks of Eppendorf AG, Germany. New Brunswick™ is a trademark of Eppendorf AG, Germany.
All rights reserved, including graphics and images. Copyright © 2016 by Eppendorf AG, Germany.