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Microcapillary Culture Method: A Novel Tool for *In Vitro* Expansion of Stem Cells from Scarce Sources

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Background and Aims. Although increasing numbers of studies report the derivation of stem cells from a variety of different tissues, derivation efficiencies greatly vary among different studies even for the same tissue source. Hence, a consistent and efficient isolation protocol has not yet been established to date. Several factors have so far been documented that influence and limit mesenchymal stem cell (MSC) isolation and cultivation, including the age and gender of the tissue donor, origin of the tissue, amount of sampled tissue material and cell culture characteristics including the choice of basal media, serum, gas composition, etc. The aim of the study was to investigate the microcapillary culture method (MCM) to establish an efficient and consistent isolation as well as cultivation protocol by comparing the results with other classic culture systems (flasks, center wells).

Methods. MSCs isolated from adipose tissue of different donors were observed comparatively under different culture systems (flasks, center wells, microcapillary tubes) and their proliferation and differentiation were investigated. Flow cytometry was used for immunophenotypic characterization of derived cells and histochemical staining (Oil Red O and Alizarin Red S) was applied for determining their differentiation capacity.

Results. It has been shown for the first time that AD-MSCs can consistently and efficiently be derived from a scarce amount of adipose tissue by MCM.

Conclusions. Further and similar studies should be performed to determine whether this methodology can also be applicable for other MSC sources. © 2012 IMSS. Published by Elsevier Inc.

Key Words: Adipose tissue stem cell, Cell culture, Microcapillary culture method, Flow cytometry.

Introduction

In recent years, long-term cell division, self-renewal, and high proliferation potency of mesenchymal stem cells (MSC) have occupied an important place in regenerative medicine, especially in tissue engineering (1).

MSCs can be derived from several sources such as bone marrow, peripheral blood, cord blood, cord matrix, dental pulp, muscle, dermis, trabecular bone, adipose tissue, periosteum, pericyte, blood, synovial membrane and liver (2). However, expensive methods are needed for isolation of SCs from these sources and it is hard to repeat isolation processes. Moreover, usually insufficient amounts of SCs are obtained by using these methods.

Adipose tissue, on the other hand, has advantages such as being easily obtained from lipoaspirates and hosting maximum amounts of MSCs per unit of the processed tissue. Owing to its aforementioned advantages, research involving adipose-derived (AD)-MSCs has recently become more popular and important in tissue engineering and regenerative medicine (3,4). Although adipose tissue is rich in terms of MSCs, similar to other stem cell sources, several factors exist that influence the efficiency of standard isolation and culture of AD-MSCs. These factors include the age and gender of the tissue donors and location of the aspirated adipose tissue (5), as well as the amount of sampled material and especially culture-dependent parameters such as the choice of the basal media, source of the serum, and gas composition (pH, pCO_2, pO_2) of the cell culture microenvironment. Although

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numerous studies have already begun to identify some of these factors and mechanisms that regulate long-term function and survival of SCs, there is still insufficient information for establishing a consistent and efficient isolation and culture methodology (6).

Several methods such as enzymatic, magnetic and selective isolation have recently been developed and used in order to increase the rate of successful derivation of AD-MSCs (7). However, increasing the amount of AD-MSCs in culture is not always proportional to the amount of processed tissue (8). Moreover, there are also other disadvantages such as being highly expensive along with the requirement of specialized technical equipment as well as qualified personnel that can make the standardization of these processes difficult for different stem cell sources.

Another important factor influencing stem cell culture is the gas phase (pH, pCO_2, pO_2) of microenvironment at a certain level (19). It has been reported that the gas phase of the stem cell microenvironment in culture affects the proliferation, division, differentiation and apoptosis of stem cells (10,11).

A previous study by Allahverdiyev et al. performed the isolation and cell culture of a very small number of eukaryotic cells in microcapillary tubes (microcapillary culture method, MCM), thereby adapting the cell culture derivation processes in a microenvironmental scale comparable to large-scale isolation and culture protocols (12,13). Whether MCM can be applicable to stem cell isolation procedures and clonal derivation of MSCs can be obtained by this novel methodology have not yet been investigated. Therefore, in this study, for the first time, the suitability of MCM for the effective isolation and cultivation of AD-MSCs were investigated by our group.

Materials and Methods

Mesenchymal Stem Cell Isolation from Adipose Tissue

Adipose tissue used for cell isolation was obtained from the lipoaspiration procedure performed during cosmetic surgery by Dr. Tunc Tiryaki (Cellest Plastic Surgery Clinic, Istanbul, Turkey). Written informed consent was obtained from six donors prior to the procedure. After transferring the lipoaspirate samples to the laboratory within 60 min, tissue pieces were washed three to four times with PBS and suspended in Dulbecco Modified Eagle Medium (DMEM) (21063-029, Gibco, Invitrogen, Grand Island, NY) supplemented with Glutamax, antibiotics, and 2 mg/mL collagenase type 2 (Sigma Chemical Co., St. Louis, MO) prewarmed to 37°C. The tissue was placed in a water bath at 37°C for 60 min, placed in hemolytic buffer for 5 min, and centrifuged at 1,200 rpm for 10 min. The pellet was harvested into DMEM supplemented with Glutamax, antibiotics (100x), and 10% FBS and incubated under conditions of 5% CO_2 humidity at 37°C. Cell culture dishes were observed daily by inverted microscope. This initial passage of the primary cell culture was referred to as passage 0 (P0) (14).

AD-MSC Culture

After P0 culture reached confluency, cells were trypsinized. Detached mononuclear cells were counted by Trypan blue staining and seeded into corresponding tissue culture vessels (Table 1) with DMEM supplemented with Glutamax, antibiotics, and 10% FBS prewarmed to 37°C, incubated under the condition of 5% CO_2 humidity at 37°C. In order to load the cell suspension into microcapillary tubes, ~60–70 μL of cell suspensions with desired cell concentration were injected into the microcapillary tubes with 1 mL injectors. The terminal ends were sealed and incubated under the condition of 5% CO_2 humidity at 37°C. After 4 days, cell culture was observed under inverted microscope (Olympus CK2, Tokyo, Japan) with 200× magnification.

Sterilization and Wax Sealing of Microcapillary Tubes

Capillary tubes were washed in a distilled water-diluted HCl solvent overnight. Then, tubes were washed under tap water about 4 h until HCl remnants leave the capillary wall. After washing with distilled water, tubes were sterilized in a Pasteur oven for 2 h at 170°C. Alternatively, prewashed and sterilized glass capillary tubes were also used for cell culture. Wax used for sealing the capillary tube terminal was heated and melted before use and placed in a glass dish. Wax is kept under sterile condition before and after use. During capillary culture preparation, tubes were taken from their sterile packages and marked. After that, cell suspension is inoculated in the tube. Both terminals of the capillary tube are covered by sterile wax and congelation takes place after a few seconds.

Cellular Expansion from Microcapillary Tubes to Center Well Dishes and 25-cm Flasks

In order to passage the cells, microcapillary culture tubes were placed in a sterile laminar hood. Both terminals of the tubes were cleaned by 70% EtOH and opened by gently breaking the terminals under sterile conditions. Media inside the tube is removed by pulling with a micropipette. After washing with sterile PBS several times, cells were dissociated by trypsin. Dissociated cells were then transferred into sterile Eppendorf tubes and centrifuged at

Table 1. Seeding densities of AD-MSCs in culture flasks, center wells and microcapillary tubes

	25-cm^2 culture flasks	Center well dishes	Microcapillary tubes
Seeding density (cells/mL)	50,000	100	100
	1,000	10	50

AD-MSCs, adipose-derived mesenchymal stem cells.

1,200 rpm for 10 min. After cell counting, cells were transferred into center well dishes (Falcon 353037, Becton Dickinson, BD Biosciences, San Jose, CA) with 2 mL of cell culture media and incubated under conditions of 5% CO_2 humidity at 37°C and observed every day. After reaching 80% confluency in center wells, cells were trypsinized and transferred into a 15-mL centrifuge tube. Cells were centrifuged at 1,200 rpm for 10 min. After cell counting, cells were seeded in 25-cm culture flasks with 5 mL of cell culture media. Culture flasks were incubated under conditions of 5% CO_2 humidity at 37°C and observed every day.

Adipose-derived Mesenchymal Stem Cell Culture and Passage

Cells in the culture flasks had a confluence of 40−50% in 3−4 days. Cells were washed twice with PBS; 10% mL of 10% FBS including DMEM was added and incubated under the same conditions. Cells were washed twice with PBS when they reached 80% confluence in the culture flasks. Trypsinized cells were centrifuged at 1,200 rpm for 10 min and passaged into three culture flasks with DMEM.

Immunological Characterization of Adipose-derived MSC

Phenotypic characterization was performed by flow cytometric analysis using AD-MSC-specific cell surface markers. Cells were maintained in 10% FBS including DMEM before analysis and labeled with antihuman antibodies: CD34-PE, and CD90-FITC (Becton Dickinson, San Jose, CA). More than 10,000 labeled cells were acquired and analyzed using a CellLab Quanta (Beckman Coulter, Miami, FL) (15).

Immunohistochemical Characterization of Adipose-derived MSC

For adipogenic differentiation, 70% confluent cultures of AD-MSCs were induced to undergo adipogenesis by replacing the stromal media with adipocyte induction medium composed of 43.2 mL DMEM, 5 mL FBS, 0.5 mL dexamethasone (Sigma-Aldich), 0.05 mL insulin (Sigma), 2 mL indomethacin (Sigma), 0.05 mL isobutylmethylxanthine (IBMX) (Sigma-Aldich), 0.5 mL penicillin/streptomycin (Sigma), and 0.5 mL l glutamine (Sigma). Cells were maintained in culture for 2 weeks, and media was replaced every 3 days. Osteogenic medium was prepared with DMEM, 0.1 μM dexamethasone, 50 μM 2 phospho-L-ascorbic acid trisodium salt, 10 mM glycerol 2-phosphate disodium salt hydrate, 1% antibiotics. In order to induce osteogenic differentiation, subconfluent cells were incubated in osteogenic medium for 2 weeks. Differentiation into chondrogenic lineage was achieved by micromass culture technique. Cell suspension, which includes 8×10^6 cells/mL, was aliquoted as 10 μl droplets in a tissue culture dish. After incubating at 37°C for 2 h for initial attachment, cells were then covered with chondrogenic medium containing DMEM, 1% FBS, 6.25 μg/mL insulin, 10 ng/mL TGFβ-1, 50 nM 2-phospho-L-ascorbic acid trisodium salt and 1% antibiotics and incubated for 2 weeks. Media was replenished every 3 days. Adipogenic, osteogenic and chondrogenic differentiation was examined by Oil Red O staining, Alizarin Red S staining and Alcian Blue staining (pH 1.0) respectively.

Oil Red O staining

After incubation, AD-MSCs were washed twice with PBS and fixed in a 10% solution of formaldehyde in PBS for 1 h. The cells were then washed with 60% isopropanol, stained with Oil Red O solution (in 60% isopropanol) for 10 min followed by repeated washing with water and destained in 100% isopropanol for 15 min. After adding hematoxylin, cells were stained for 1 min at room temperature and washed again with water (16). Inverted microscopy was used for determination of the differentiation.

Alcian Blue staining

For Alcian Blue staining, after fixing the cells with 4% PFA solution, proteoglycans in the chondrogenic tissue were stained with 0.1 N HCl containing 1% Alcian Blue for 30 min.

Alizarin Red S staining

Osteogenic differentiation was performed according to a previously published protocol (17). Cells were washed twice with PBS. Alizarin Red S stain was added and stained for 2−5 min at room temperature. After removing the stain, cells were fixed with acetone. After removing acetone, acetone/xylene mixture was added to the cells. Finally, cells were fixed by xylene. Cells were dried at room temperature, covered with glycerol and observed under microscopy.

Results

Isolation and Culture of AD-MSCs

Cells isolated from the lipoaspirate sample by enzymatic digestion were adhered to the tissue culture flask and left for proliferation. Nonadherent cells, such as red blood cells, were removed by replacing the medium. After 24 h of seeding, initial adherent cells with spindle- or satellite-shape were observed in culture. These cells generated visible colonies 3−4 days after the initial plating. For the next step, a gradual cell proliferation system was developed for AD-MSC culture. Also, gradual cell culture was investigated and cell proliferation in microcapillary tubes and center wells was determined (Table 1).

Effect of Cell Amount in Flasks for AD-MSCs (50,000 cells/mL)

Cells were seeded in such a way that there were ~50,000 cells/mL in 25-cm^2 culture flasks. Cells attached culture

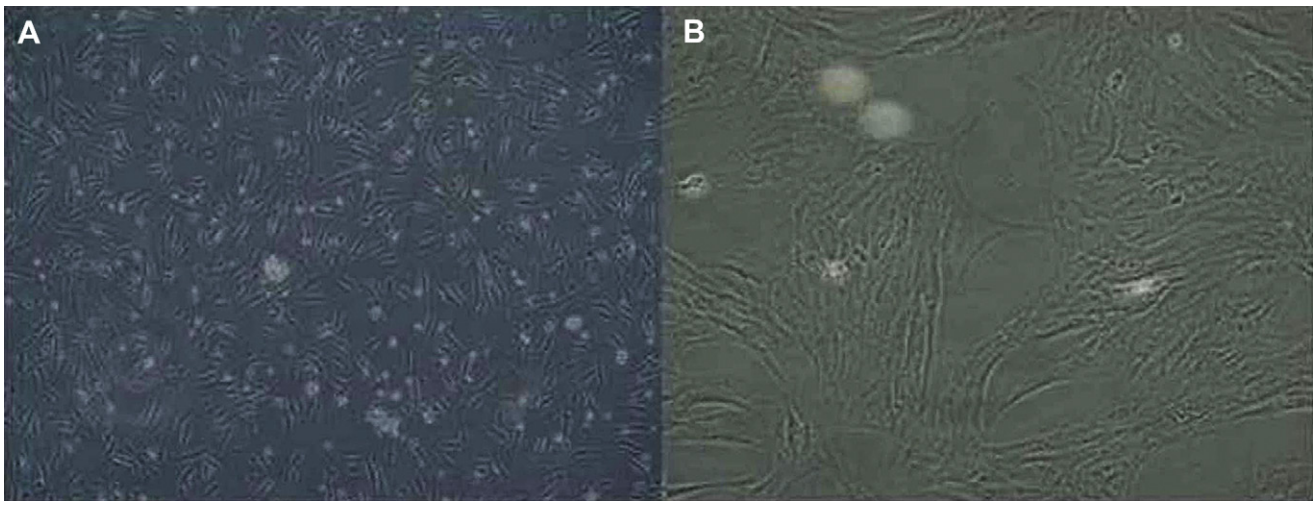


Figure 1. Morphology of adipose tissue-derived mesenchymal stem cells (AD-MSCs). Spindle-like appearance of AD-MSCs in culture flasks. (A) Six days after initial plating (magnification ×20). (B) 16 days after initial plating (magnification ×40).

flask surfaces within 24 h. Cells were observed every day under inverted microscope. Initial cells grew as spindle- or satellite-shaped cells after first and second passages and proliferated. Until the second passage, there was a heterogeneous cell culture as reported in the literature (18) and nonadaptive cells were replaced by refreshing the media. Thus, a homogeneous cell culture was obtained after the third and/or fourth passages (Figure 1A). Cells continued to proliferate quickly.

Effect of Cell Amount in Flasks for AD-MSCs (1,000 cells/mL)

After isolation, cells were diluted 50-fold and seeded in 25-cm^2 culture flasks. Cells attached to the flask surface after 24 h. Cells were observed every day under inverted microscope. Initial cells grew as spindle- or satellite-shaped cells but they did not proliferate to confluency and started to degenerate during extended culture. There were granules observed within the cytoplasm of the cells and cytoplasmic extensions began to rupture. Granules around the nucleus were also evident. It is concluded that cell culture could not maintain normal proliferation with the dilution of 1,000 cells/mL in 25-cm^2 flasks. Eventually, cells detached from flask surfaces during prolonged culture (Figure 1B).

Effect of Cell Density in Center Well Dishes for AD-MSCs (100 cells/mL)

After isolation, cells were seeded into center wells (~3.14-cm^2 cell growth area) with a ratio of 100 cells/mL with 2 mL of media. Cells started to show stem cell morphology after 1 night incubation and attached to the bottom of the flask. Microscopic observation showed that cells started to proliferate after 24 h. Cells were intact in the center well. Additionally, cells were observed to adapt

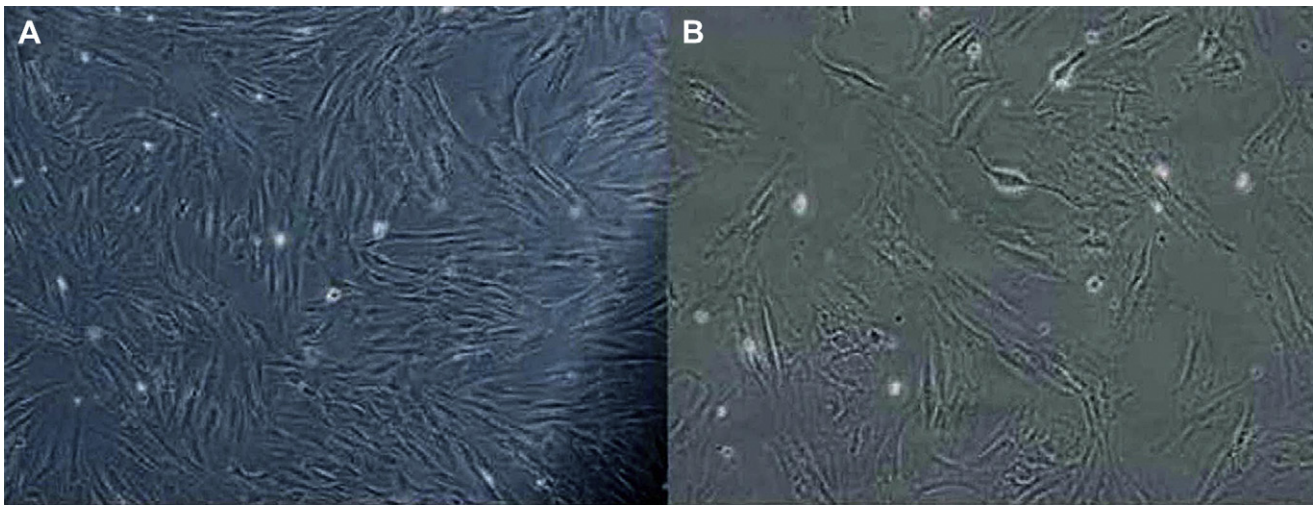


Figure 2. Morphology of AD-MSCs in center wells. (A) 8 days after initial plating (magnification ×20). (B) 16 days after initial plating (magnification ×40).

to center well conditions and 80% confluency was reached in about 7−10 days (Figure 2A).

Effect of Cell Density in Center Well Dishes for AD-MSCs (10 cells/mL)

After isolation, cells were seeded into center wells with a ratio of 10 cells/mL with 2 mL of media. Cells started to show stem cell morphology after 1 night incubation. Daily microscopic observation results showed that cell amount did not increase significantly. It was observed that cells did not adapt to the conditions and started to degenerate by forming cytoplasmic fragmentations. Furthermore, detachment of cells was seen in 14- to 23-day cultures (Figure 2B).

Effect of Cell Amount in Microcapillary Tubes for AD-MSCs (100 cells/mL)

AD-MSC culture was achieved by microcapillary culture method with the need for less expensive and less media. Cells attached to the tube walls at a rate of 100 cells/mL in 60−70 μL media after 24 h. Proliferating cells were observed by microscopy (Figure 3).

Effect of Cell Amount in Microcapillary Tubes for AD-MSCs (50 cells/mL)

Cells were seeded in microcapillary tubes in 60−70 μL of media containing 50 cells/mL. After 1 night incubation, cells attached to the tube walls and exponential proliferation was observed (Figure 3B).

Expansion of Microcapillary Culture-derived AD-MSCs

Proliferated cells in microcapillary tubes were transferred into center wells on the 11th day of the cultivation. Cells were trypsinized and transferred into center wells (Figure 4). After 2−4 h of seeding it was observed that cells started to adhere on the center well bottom. After 5−6 days of culture, cells were trypsinized again and passaged into two center well dishes. After confluency was reached, proliferated cells in center well dishes were again trypsinized and passaged into 25-cm^2 culture flasks and so gradual expansion and adaptation of cells from microcapillary tubes to culture flasks were obtained.

Adipogenic, Osteogenic and Chondrogenic Differentiation

After cultivation in control medium, AD-MSCs were cultured by adipogenic medium for 14 days for adipogenic differentiation. In adipogenic medium, cells acquired expanded cell morphology, and a time-dependent increase was observed in intracellular lipid vacuoles. These lipid vacuoles became visible after 4 days of adipogenic induction, and the cell size and number of lipid vacuoles increased incessantly afterwards. Adipogenic differentiation potentials of AD-MSCs were detected by Oil Red O staining (Figures 5A and 5B). Osteogenic differentiation was induced by osteogenic medium. Cells started to show crystal-like nodules after 22-day culture. Osteogenic differentiation potentials of AD-MSCs were detected by Alizarin Red S (Figure 6). Likewise, after induction for 2 weeks, chondrogenic differentiation was observed and documented by Alcian Blue staining (Figure 7).

Phenotypic Characterization of AD-MSCs

The immunological characterization of undifferentiated human AD-MSCs at passage 5 was examined by flow cytometry. We characterized the AD-MSC population according to its cluster of differentiation (CD) marker profile using flow cytometry. AD-MSCs were found to express CD90 (95.97%) but, as expected, they were not found to express hematopoietic lineage markers such as CD34 (1.06; data not shown).

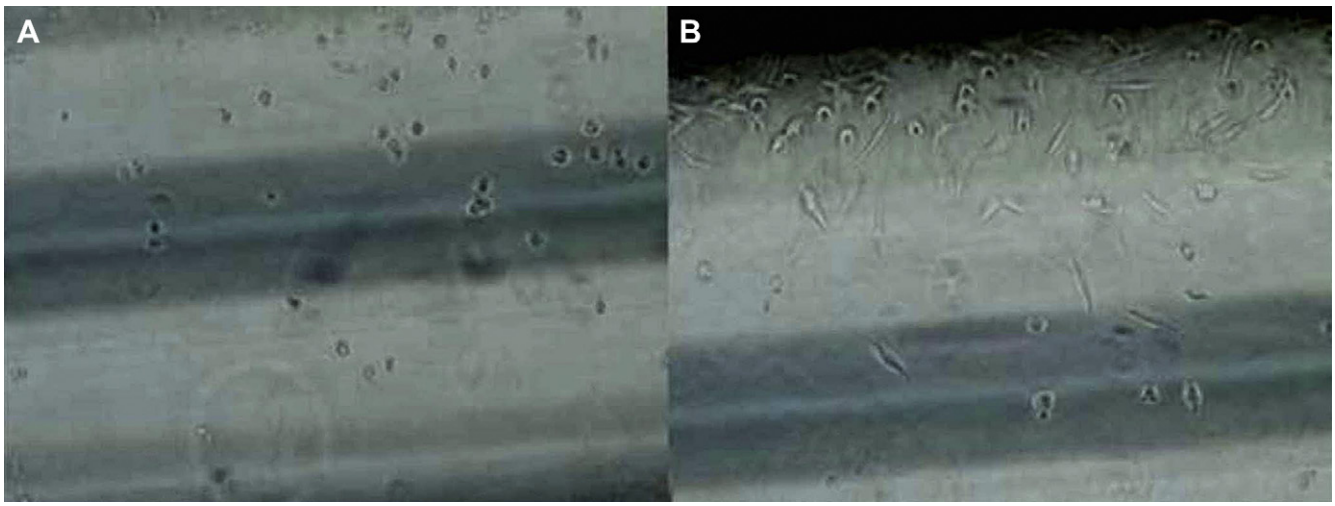


Figure 3. Microscopic observation of AD-MSCs in microcapillary culture. (A) 1 h after initial plating. (B) 48 h after initial plating (magnification ×40).

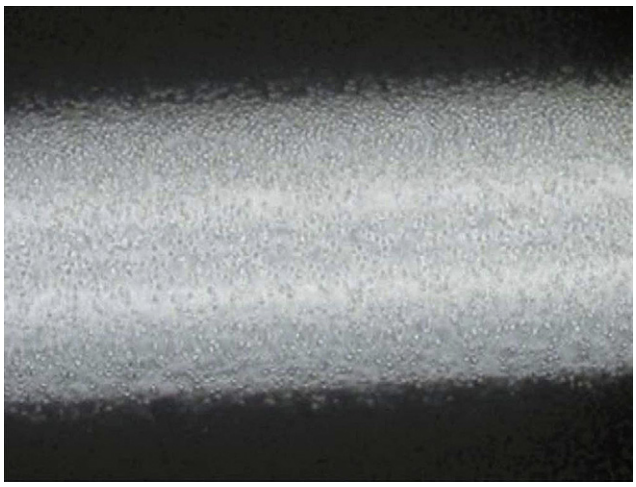


Figure 4. Cell density in microcapillary tubes during trypsinization. 11 days after initial plating (magnification ×40).

Cell Cycle Analysis

After 48 h of AD-MSC subculture, cell cycle analysis was achieved by flow cytometry: 42.27% of cells were at G0/G1 phase, 4.6% of the cells were at S phase and 9.39% of the cells were at G2 phase. Cell cycle analysis results showed that most of the cells were at G0 phase, and it was supported by the other MSC cell cycle results (19).

Discussion

To date, there are several culture systems applied for human and animal AD-MSC culture. However, these culture systems differ from each other according to several characteristics including culture volume, cell adhesion properties of the surface area, gas phase level, etc. Apart from the scarcity of the donor tissue, all of these factors can potentially affect successful MSC isolation and culture. Unfortunately, no universal and consistent isolation and culture protocol exists that can be applied for stem cell biology.

For this reason, in this study the efficiency and applicability of MCM in stem cell isolation and expansion was investigated for the first time. AD-MSCs were chosen as the primary stem cell source because it is easier to obtain large amounts of adipose tissue in order to use the extracted cells in different arms of the study. Cells obtained from one donor were sufficient to use in all of the culture systems, making the cell culture comparisons as homogeneous as possible in order to minimize the interindividual effect.

MCM was developed by Allahverdiyev et al. and has been previously applied in our laboratory for the culture of limited numbers of eukaryotic cells effectively (data not shown) (12). This method is based on the culture of cells in a CO_2-rich microaerophyl environment so that this environment can supply the optimal conditions for cellular adaptation and proliferation.

According to the results, it is concluded that the MCM can be a suitable method for initial proliferation and as a first step during *in vitro* expansion. Moreover, comparing the need for prolonged cell culture in conventional isolation methods, using the MCM in the initiation phase makes the whole procedure relatively economical for scarce sources. Much less cell culture media, nutrients and less equipment were sufficient for gradual proliferation and expansion of AD-MSCs in our experiments.

The effect of microaerophyl condition on cell proliferation is also supported by other researchers. Balin et al. reported that oxygen level of cell culture effects toxicity on cells because reactive oxygen species (ROS) are changing

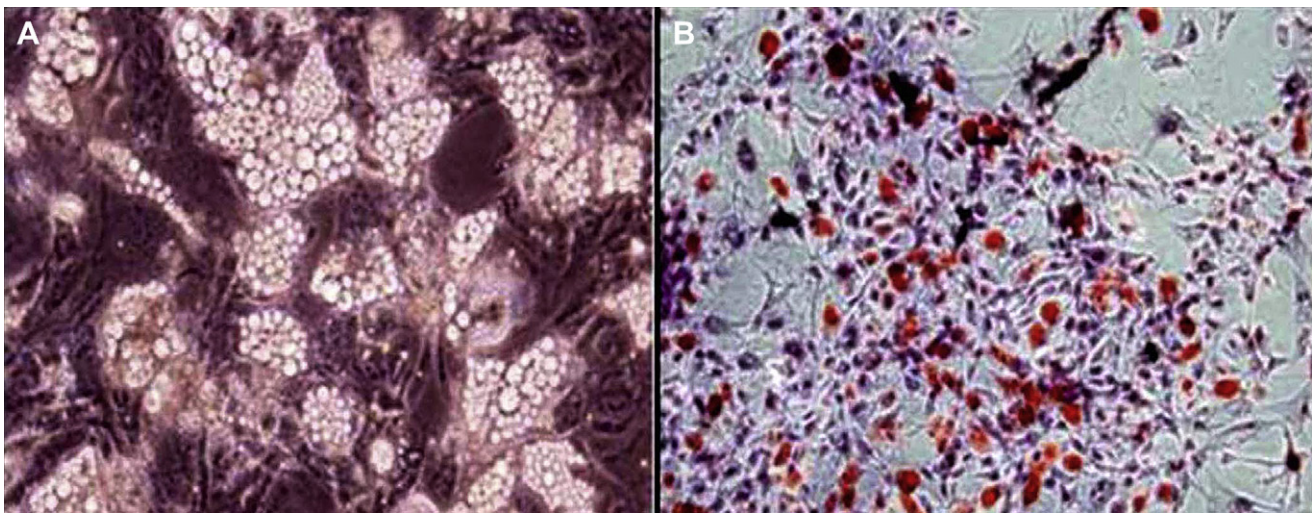


Figure 5. Adipogenic differentiation. (A) Lipid vesicles seen 16 days after induction of differentiation (magnification ×40). (B) Same culture after Oil Red O staining (magnification ×20).

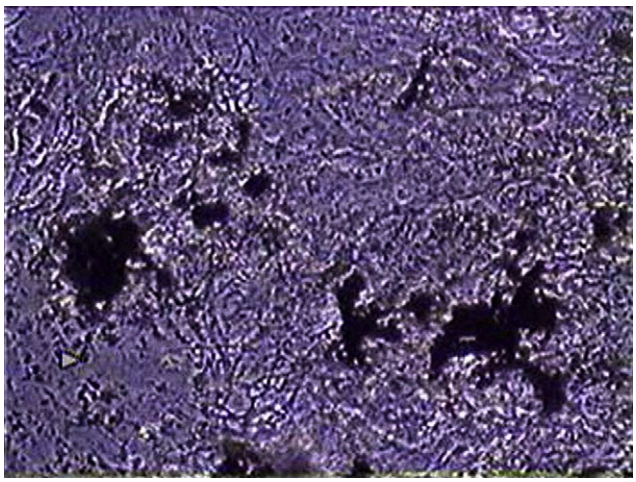


Figure 6. Osteogenic differentiation of AD-MSCs 14 days after induction of differentiation (magnification ×40).

the pH levels (9). In microcapillary culture tubes, O_2 level is low and CO_2 level is high and pH generally decreases approximately on average of 0.1 U. Therefore, ROS production reduces and inhibits the toxic effect. On the other hand, secreted proteins are important for cell proliferation and for retaining the microenvironmental conditions. Also, it is reported that except cell culture flasks and cell culture media, secreted proteins of cells and protecting their microenvironment conditions are important and effective on proliferation, survival, apoptosis, necrosis or differentiation (10). Otherwise, stem cell adhesion area in center well is 3.14-cm and also ~2 mL media is needed for cell proliferation. This increases the interaction between cells and the external environment. Furthermore, the center well is a small radius culture dish where gas exchange occurs and has less cell adherence area than microcapillary culture tubes.

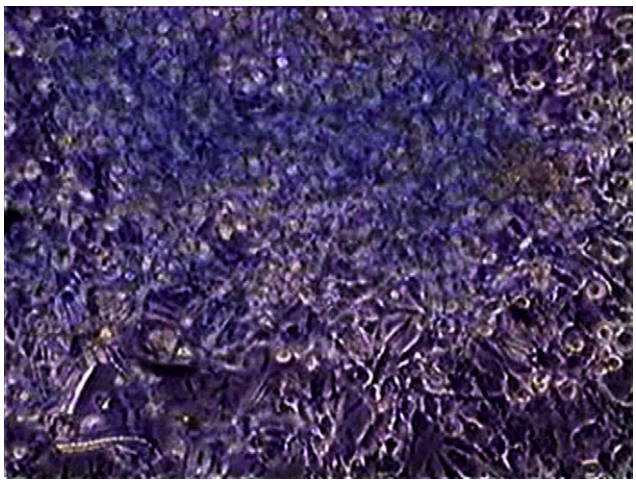


Figure 7. Results of Alcian Blue staining for chondrogenic differentiation (magnification ×40).

Microcapillary culture tubes have a 0.9-cm cell adherence area and require 60−70 μL of cell culture media. This means that the required cell surface adherence area and media volume ratio is 8−10 times lower than other culture dishes. Therefore, it is thought that fewer stem cells can be maintained in microcapillary culture tubes with 8- to 10-fold fewer media than center wells or such two-dimensional culture dishes, and cells can proliferate in this microaerophyl environment rich from cell metabolites and secreted proteins.

In conclusion, for the first time it has been shown in this study that it is possible to proliferate and expand scarce numbers of AD-MSCs gradually using the MCM as the initial step. According to our results, another most important advantage of this method is that the method can increase the rate of successful isolation by facilitation during the initial adaptation phase. Hence, shorter incubation period and fewer culture materials are needed for initial passages, making the isolation process cost-effective as compared to contemporary protocols. The authors believe that the MCM method can be an effective isolation and expansion protocol for stem cell isolation procedures in tissues where the tissue volume and the numbers of stem cells within the tissue are very limited such as cord blood.

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Disclosure

The authors have no relevant affiliations or entity with a financial conflict with the subject matter or materials in the manuscript.

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