

The growth kinetic, differentiation properties, karyotyping, and characterization of adipose tissue-derived stem cells in hamster

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Abstract Multilineage properties of mesenchymal stem cells (MSCs) cause their contribution in repair of different tissue. Adipose tissue is considered a good source of MSCs because of its abundance and easy access. The aims of this study were to characterize and determine the differentiation properties and growth kinetics of adipose tissue-derived stem cells (AT-SCs) from Syrian hamster. Fat tissue was isolated from the abdominal and pelvic regions of the hamster and by using type I collagenase, and cells were isolated and cultured. AT-SCs were characterized by morphology, cell surface markers, and adipogenic and osteogenic differentiations. AT-SCs were transferred to 24 well plates, and the growth kinetic was assessed by evaluation of the population doubling time (PDT). To show the chromosome stability of the AT-SCs, karyotyping was done. Hamsters' AT-SCs showed fibroblast like morphology and expressed CD73 and CD29 but not CD45. AT-SCs could be differentiated to adipocytes and osteoblasts verified by Alizarin Red and Oil Red O staining methods. The PDT of the passages 2 and 3 of the AT-SCs were 51.6 and 80.6 h, respectively. The karyotyping was normal until passage 4. Hamsters' AT-SCs showed mesenchymal properties denoting to these cells as a good source for the cell transplantation purposes in experimental and preclinical studies in hamster as an animal model.

Keywords Mesenchymal stem cells · Adipose tissue · Growth kinetics · Differentiation · RT-PCR

Introduction

In recent decade, mesenchymal stem cells (MSCs) were introduced in experimental and clinical applications in various species (Ghobadi et al. 2015). The important properties of MSCs including potential of multilineage differentiation and extensive ability of self-renewal made them the best choice for cell therapy of different disease (Mehrabani et al. 2016a). Adult mesenchymal stem cells have been isolated from different sources such as bone marrow (Tamadon et al. 2015), dental pulp (Mahdiyar et al. 2014), umbilical cord (Ding et al. 2015), endometrial tissue (Mehrabani et al. 2015b), menstrual blood (Mehrabani et al. 2016b), and adipose tissue (Mehrabani et al. 2015a).

Adipose tissue-derived stem cells (AT-SCs) have been attracting as a main source for cell therapy, because of low cell yield and the painful isolation process of BM-SCs (Kisiel et al. 2012). They are a suitable, abundant, accessible, highly proliferative, and rich source of MSCs (Baer and Geiger 2012; Mehrabani et al. 2013; Peng et al. 2008).

AT-SCs were first extracted and characterized in rat by Rodbell (1966). In addition, beside proliferation capacity of AT-SCs, their differentiation potential is the other important aspect in application of AT-SCs in cell therapy and tissue engineering (Baghaban Eslaminejad et al. 2008). AT-SCs plasticity and ability to differentiate into mesodermal tissues such as bone, fat, and cartilage are considered as hallmark criteria for AT-SCs classification (Larocca et al. 2013). Furthermore, AT-SCs express the surface mesenchymal lineage markers CD29, CD44, CD73, CD90, and CD105, while the markers of hematopoietic cells including CD11c, CD31,

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CD34, CD45, CD80, and CD86 were not detected on AT-SCs (Mosna et al. 2010).

MSCs derived from hamster as an animal model have been used in heart failure (Shabbir et al. 2009b), muscular dystrophy (Shabbir et al. 2009a), and azoospermia (Tamadon et al. 2015). Therefore, the goals of the current study were to isolate, culture, and characterize hamster AT-SCs for their growth kinetics, differentiation capacities, and karyotyping. Furthermore, we analyzed AT-MSCs differentiation potential to osteogenic and adipogenic cell lineages.

Materials and methods

Animals

Mature female Syrian hamsters weighing 90–130 g were provided from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Science, Iran. They were housed under 12 h light and 12 h dark cycle in a temperature-controlled room (20–22 °C). Tap water and standard laboratory chow were available for all animals ad libitum. They were housed and euthanized according to the instructions of the Animal Care Committee of Shiraz University of Medical Sciences.

Isolation and culture of adipose tissue cells

Hamsters were anesthetized by ether and under anesthesia were euthanized by cervical dislocation. Fat tissue was isolated from the abdominal and pelvic regions of the hamsters. After cutting the adipose tissue, they were transferred into sterile phosphate buffered saline (PBS) in falcon tubes. The tissue samples were chopped using a scalpel blade in small pieces. They were transferred into 0.2 % collagenase type II (Gibco, USA) at 37 °C for 40 min for digestion. Then, by adding 5-ml Ham's F-12 cell culture medium (Biowest, France) containing 10 % fetal bovine serum (FBS; USA), 1 % penicillin and streptomycin (Gibco, USA), and 2 % L-glutamine (Invitrogen, Netherlands), the enzyme activity was stopped and the isolated cells were separated from debris using cell strainer (70 µm).

The suspension was centrifuged at 1500 rpm for 5 min and after removing the supernatant, the pelleted fraction was re-suspended in 5-ml Ham's F-12 complete medium. The suspension was transferred into 25-ml flasks. The flasks were incubated in a 5 % CO₂ incubator at 37 °C and saturated humidity. The initial passage of the primary cell culture was referred to as "passage 0". Changing the medium, non-adherent cells were removed after 24 h and fresh medium was added again. The culture medium was replaced every 3 days. In 80–90 % confluency, the cells were harvested using

0.25 % trypsin (Gibco, USA), counted and subcultured into new flasks.

Cell freezing and thawing

The isolated cells in passage 3 were cryopreserved. The cells were suspended at a density of 2×10^6 viable cells per milliliter in freezing media including 10 % dimethyl sulfoxide (DMSO; MP Bio, France) and 90 % FBS. The cell suspension was divided into sterile plastic cryovials that were labeled with the date and the freezing serial number. The vials were kept at –20 °C for 60 min and then they were transferred to –70 °C for 24 h. Finally, for long-term storage, cryovials were transferred into liquid nitrogen.

For subculturing after removing from the liquid nitrogen, the cryovials were quickly thawed in a 37 °C water bath. When the ice clump was almost thawed, 1 ml of Ham's F-12 complete medium was added and centrifuged at 1500 rpm for 5 min. The cells were re-suspended and transferred into a flask gently blown into uniform single cell suspension and incubated at 37 °C and 5 % CO₂. All flasks were subcultured just up to passage 4. Cells were subcultured similarly up to 4th passage (P4), and at the end of each passage, the live and dead cells were enumerated by a Neubauer chamber and staining the cells by trypan blue (Sigma, USA).

Expression of AT-SCs markers

To assess the expression of mesenchymal specific markers (CD29 and CD73) and hematopoietic specific marker (CD45), RT-PCR was performed (Table 1). The total RNA was extracted according to the manufacturer's instructions by a Column RNA Isolation Kit (Denazist-Asia, Iran). Total RNA concentration was evaluated by spectrophotometer. From RNA samples, the cDNAs (complementary DNAs) were synthesized according to the manufacturer's instructions by AccuPower Cycle Script RT PreMix Kit (Bioneer, Korea). Briefly, total RNA was used for each reaction and 12 thermal cycles was performed: primer annealing at 20 °C for 30 s, cDNA synthesis for 4 min at 42 °C, melting secondary structure and cDNA synthesis for 30 s at 55 °C. Finally, heat inactivation was performed for 5 min at 95 °C. In the third step, the microfuge tubes containing 1 µL of template (cDNA) were mixed with other reagents consisting of dNTPs, H₂O, MgCl₂, PCR buffer, Taq DNA polymerase, and forward and reverse primers (CD29, CD73, and CD45) were put in Thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). Accordingly, 30 amplification cycles were run consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 64 °C, extension for 30 s at 72 °C, primary denaturation for 2 min at 95 °C, and final extension for 5 min at 72 °C. To evaluate the presence of considered bands by gel electrophoresis, the PCR products were analyzed with the aid of DNA

Table 1 The primers used for RT-PCR analysis of mesenchymal cell confirmation of adipose tissue-derived mesenchymal stem cells in hamster

Primer	Direction	Primer sequence	Amplicon length (bp)
CD45	Forward	TGA TGC TAT GCT GGA AGG	265
	Reverse	GTA TGA AGG AAG TCT CTG GAA	
CD73	Forward	TGG GAG GGA GAA GAG GAT GGC	336
	Reverse	GGA GAG TGG CAG GCA GAA ATA AAC C	
CD29	Forward	CAG TGA ATA GCA ACA ATG AAG C	133
	Reverse	ATA AGC AGC AAG GCA AGG	

safe stain in 1.5 % agarose gel medium and were visualized by Gel documentation system (UVtec, Cambridge, UK).

Adipogenic and osteogenic differentiation assay

In order to evaluate the differentiation potential of AT-SCs into adipocytes, cells at 90 % confluence were cultivated in Ham's F-12, 15 % FBS, 0.2 mM L-glutamine, 100 μ M L-ascorbic acid (Merck), 200 μ M indomethacin, and 100 nM dexamethasone. The medium was changed biweekly for 3 weeks. Adipogenic differentiation was evaluated using Oil Red O staining after 21 days. For evaluating the generation of oil droplets, the cells were fixed in 10 % formalin for 10 min at room temperature and were washed twice with water. Oil red O working solution was prepared by mixing 4-mL distilled water to 6-mL stock solution (0.5-g oil red in 100 mL isopropanol) and was filtered by Whatman filter paper. Then, Oil Red O stain was added into fixed cells and incubated for 1 h at room temperature. Finally, the stained cells were rinsed with water several times and were observed under an inverted microscope (all non-referred reagents from Sigma-Aldrich, USA).

For osteogenic differentiation of AT-SCs, cells of passage 3 were seeded in 6-well plates. After the cells reaching 70 % confluency, they were cultured for 3 weeks in osteogenic medium containing low glucose DMEM supplement with

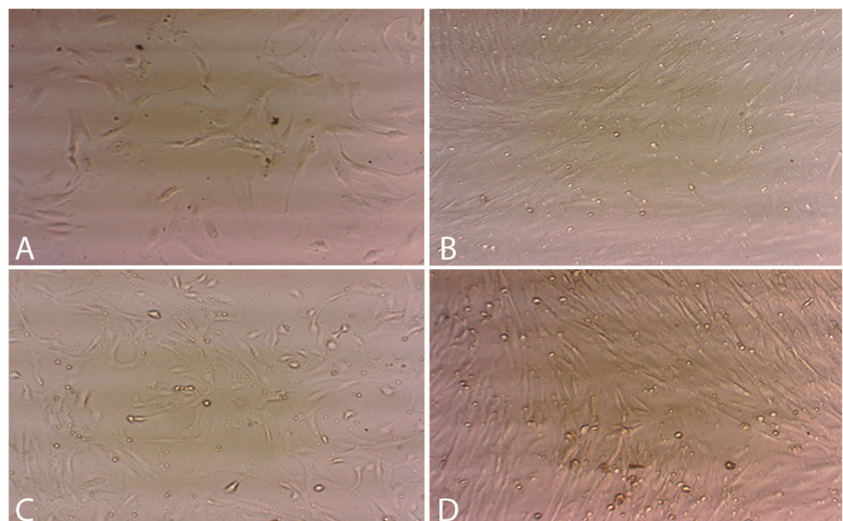
0.05 mM ascorbate-2-phosphate (Wako Chemicals, Richmond, VA, USA), 10 mM b-glycerophosphate, 100 nM dexamethasone, 10 % FBS, and 1 % antibiotic/antimycotic. The medium was replaced every 3 days. At day 21, the cells were fixed by 10 % formalin solution, and then stained using Alizarin Red to detect calcified extracellular matrix and osteogenic differentiation. A control culture of AT-MSCs was stained using Alizarin Red without pre-exposure to osteogenic media (all non-referred reagents from Sigma-Aldrich, USA).

Growth kinetics

The growth curves were plotted for MSCs derived from abdominal adipose tissue in order to better compare growth kinetics of the cells. For the assessment of growth characteristics, AT-SCs at passage 2 and 3 were seeded in a 24-well plates at a density of approximately 5×10^4 cells per well in triplicate. Cells were collected from each well on days 1 to 7 after seeding and counted by light microscope to draw cell growth curves. The curves were drawn using GraphPad Prism (version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

To compare the in vitro proliferation rate, the PDT value was determined for cells in both passages. PDT was calculated using the formula $PDT = T \ln 2 / \ln (X_e / X_b)$, in which T is the incubation time in hours, X_e represents to the cell number at

Fig. 1 Morphological characteristics of adipose tissue-derived stem cells (AT-SCs) of hamster. MSCs showed fibroblastic morphology. **a, b** Passage 2 of AT-SCs ($\times 100$ and $\times 200$). **c, d** Passage 3 of AT-SCs ($\times 100$)



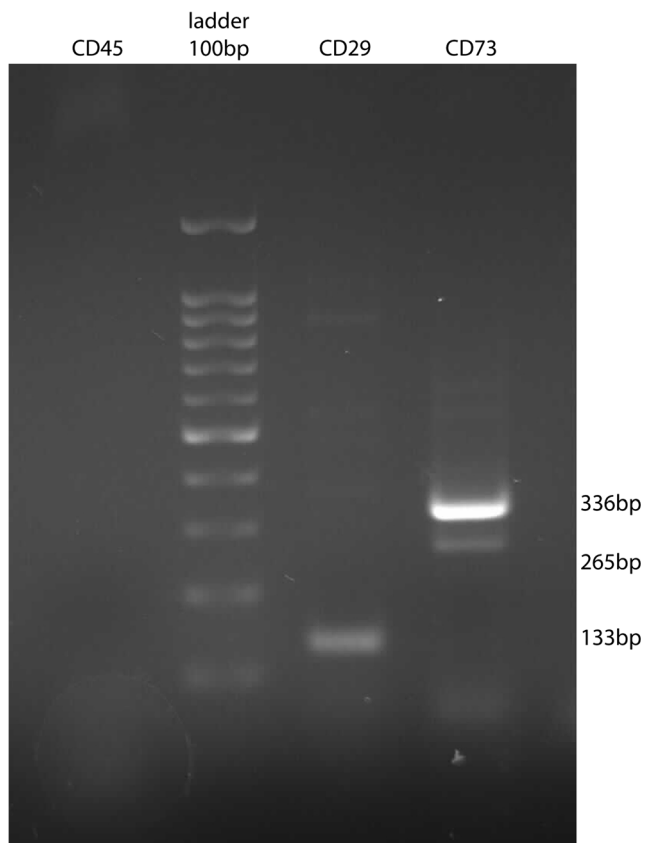
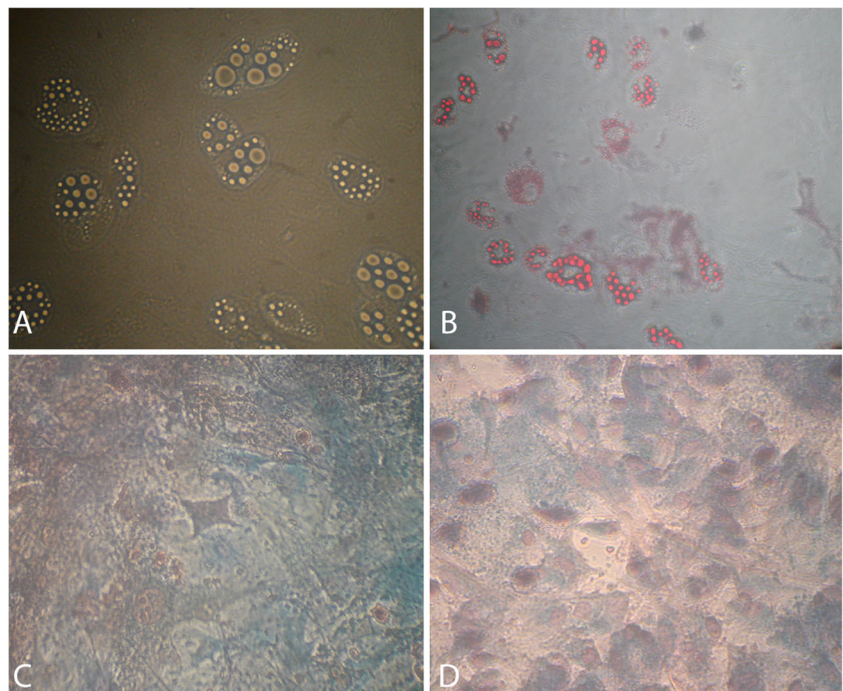


Fig. 2 Agarose gel electrophoresis of RT-PCR products of adipose tissue-derived stem cells of hamster show positive expression for CD29 and CD73 (mesenchymal surface marker) and negative expression for CD45 (hematopoietic surface marker)

Fig. 3 Differentiation of adipose tissue-derived stem cells (AT-SCs) into adipocytes before (**a**, $\times 200$) and after (**b**, $\times 100$) Oil Red staining. **c** Osteogenic differentiation of AT-SCs from passage 3 of subcultures by Alizarin Red staining ($\times 100$). **d** Control AT-SCs without osteogenic differentiation after staining by Alizarin Red ($\times 100$)



the end of incubation time, and Xb corresponds the cell number at the beginning of the incubation time.

Karyotype

Karyotyping cells that were subcultured at a 1:3 dilution (both the primary culture and after the last passage) were exposed to a 4 h demecolcemide (Sigma, USA). After 24 h, trypsin-EDTA (Sigma, USA) and hypotonic KCl (Sigma, USA) were added and then fixed in an acid/alcohol solution. The number of chromosome was counted by a microscope using an oil immersion objective. Images were provided using a digital camera on a light microscopy for analysis of the metaphase.

Statistical analysis

The means and standard error of means (SE) of counted cells in the growth curve analysis were compared using one-way ANOVA (SPSS for Windows, version 11.5, SPSS Inc., Chicago, USA) and Tukey post-hoc test. Values of $P \leq 0.05$ were considered significant.

Results

Cell morphology

After 48 h, fibroblastic-like cells were attached to the tissue culture flask (Fig. 1).

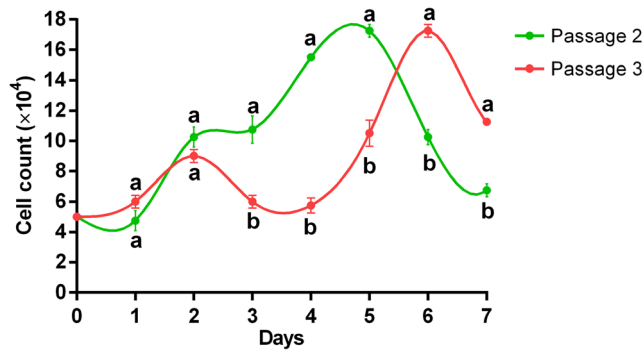


Fig. 4 Comparison of mean and standard error of cell counts between growth curves of hamster adipose tissue-derived stem cells at passages 2 and 3. ^{a,b}Superscript letters show differences between the number of cells in each curve in the same day ($P < 0.05$)

AT-SCs markers

AT-SCs were positive for MSC markers of CD29 and CD73 and negative for hematopoietic marker of CD45 (Fig. 2).

Differentiation assay

The AT-SCs were differentiated into adipocytes and osteoblasts (Fig. 3). The negative control (non-induced) cells for each type of differentiation were negative for Alizarin Red and Oil Red O stains. Osteogenesis of AT-SCs demonstrated mineralized matrix 21 days after induction. Adipogenesis of AT-SCs was detected by the formation of lipid droplets stained with Oil Red O staining, 21 days after induction.

Growth kinetics

PDT of the passages 2 and 3 of the AT-SCs were 51.6 and 80.6 h, respectively. By comparing of growth curves, proliferation rate of cells in passages 2 was more than passage 3 (Fig. 4; $P < 0.05$).

Karyotyping

Karyotype analysis of the cells in passage 4 revealed a chromosomal number of $2n = 44$ including one pair of sex

chromosomes and 21 pairs autosomal chromosomes. A normal diploid karyotyping of AT-SCs was confirmed as illustrated in Fig. 5.

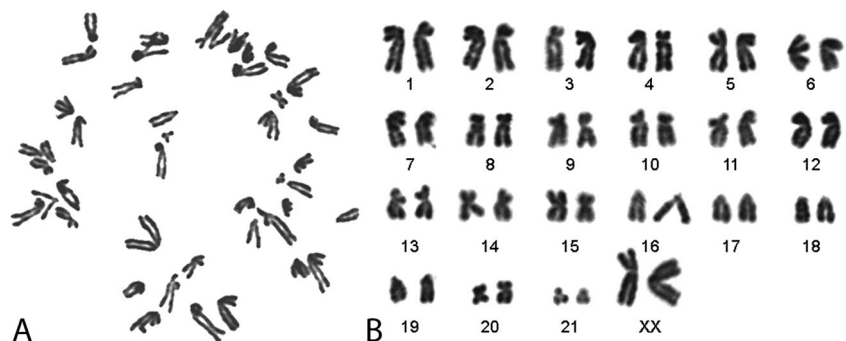
Discussion

In the present study, adhesion of cells to the plastic tissue culture flasks was noticed and the cells had fibroblast like morphology similar to previous studies confirming the mesenchymal property of cells (Aliborzi et al. 2015, 2016; Mehrabani et al. 2015a). Evaluation of the cell markers of CD73, CD29, and CD45 at passage 4 was done by RT-PCR showing that the AT-SCs were positive for CD73 and CD29 and negative for CD45. These markers confirmed the mesenchymal nature of the isolated cells as described before (Baer and Geiger 2012; Mehrabani et al. 2013; Peng et al. 2008). Adipose tissue contains a source of stem cells that could be easily isolated, is easily accessible and available by noninvasive methods, and is easily expandable to millions of cells without significant changes in phenotype and genotype (Baer and Geiger 2012; Mehrabani et al. 2013; Peng et al. 2008).

According to the growth curve analysis, growth and proliferation rate of AT-SCs decreased gradually from passage 2 to passage 3. Consistent with our results, with increasing passage number of the human and Guinea pig MSCs, proliferation, growth rate and the number of cells in culture decreased in higher passages (Aliborzi et al. 2016; Bruder et al. 1997; Kang et al. 2004). The PDT of AT-SCs in passages 3 were more than 2. Peng et al. (2008) reported a PDT of 45.2 h for AT-SC in human in passage 3. The origin of cells, the cultivation conditions, and difference in medium supplements may affect the PDT of MSCs (Schipper et al. 2008).

In addition to proliferation potential, differentiation capacity has been considered as an important quality of MSCs. Differentiation is a process which dramatically influences the cell size, morphology, membrane potential, and metabolic activity due to highly-controlled modifications in gene expression (Alipour et al. 2015). According to Alizarin Red staining for mineralized matrix in osteogenic differentiation and Oil Red staining for lipid droplets in osteogenic differentiation,

Fig. 5 Chromosomes at metaphase (a) and karyotype (b) of adipose tissue-derived stem cells (AT-SCs) of hamster after passage 4. The karyotype of the AT-SCs of hamster consisted of 22 pairs of chromosomes. The sex chromosome type was XX (♀)



AT-SCs differentiated into adipose and bone cell lineages that were previously reported in other studies (Chen et al. 2016; Mehrabani et al. 2015a; Wagner et al. 2005).

In conclusion, as AT-SCs are an accessible source of MSCs, they are readily expanded in culture flasks, have a good proliferation capacity, and display high potential toward adipogenic and osteogenic differentiation, they can be considered as an efficient source of MSCs and an appropriate candidate for use in preclinical studies and regenerative medicine applications.

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Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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