Full Length Research Paper

High yield generation of hepatocyte like cells from adipose derived stem cells

Abbas Sahebghadam Lotfi[1]*, Alireza Khoshdel[1], Masoud Soleimani[2], Morteza Daliri[3], Vahid Razban[3] and Behzad Adibi[1]

[1]Departments of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, P. O. Box 14115-111, Tehran, Iran.
[2] Departments of Hematology, Faculty of Medical Science, Tarbiat Modares University, P. O. Box 14155- 6343, Tehran, Iran.
[3]National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

Mesenchyma stem cells (MSCs) are multipotent cells. They are capable of transforming into several mesoderm lineages. They can be separated from different tissues, such as bone marrow, umbilical cord blood, peripheral blood, and adipose tissue. Stem cell based therapies for the repair and regeneration of individual tissues and organs suggest a paradigm shift which may supply optional therapeutic solutions for a number of diseases. Embryonic and induced pluripotent stem cells have hypothetically been beneficial, although there are various restrictions. Bone marrow-derived mesenchyma stem cells when compared to adipose-derived stem cells (ADSCs), they were obviously capable of differentiating into cells and tissues with mesodermal origin. Adipose tissue is prevalent and easily available in large quantities. The intent of this investigation was the isolation of mesenchymal stem cells from rat adipose tissue, the potential of MSCs in differentiating into osteogenic and adipogenic lineages, describe the ADSCs surface expression of the classic markers by using flow cytometric analysis, and their transformation to hepatocyte like cells. Immunocytochemical analysis displayed that ADSCs express albumin and α-fetoprotein during differentiation. Knowledge of these cells may also demonstrate many benefits in cell-based therapies for tissue repair, regeneration, or tissue engineering.

Key words: Hepatocyte-like cells, mesenchymal stem cells, cell therapy and adipose derived stem cell.

INTRODUCTION

Although, embryonic and induced pluripotent stem cells have hypothetically highly beneficial, there are various restrictions imposed by cell directions, ethical considerations, and genetic manipulations (O'Donoghue, 2004). Several criteria have been considered for perfect clinical reviving medicine using stem cells including; abundance in quantities (millions to billions cells), minimally intrusive procedures of harvesting,

differentiation along multiple cell lineages path in a regulation and reproducible manner, safe and effective transplantation to either an autologous or allogeneic host and can be produced in accordance with current good manufacturing practice recommendations. Adipose tissue could fulfill all these criteria. Adipose-derived stem cells (ADSCs) can be isolated from the mesenchyme and differentiated to other tissues, such as bone marrow (Gronthos et al., 2001). These cells have self-reviving skill and they can differentiate into varied mesenchymal tissues, including chondrocytes, adipocytes, osteoblasts, myocytes and endothelial cells (Zuk et al., 2002; Planat et al., 2004; Bianco et al., 2001). ADSCs have similar specificities with bone marrow-derived Mesenchymal

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*Corresponding author. E-mail: Lotfi-ab@nigeb.ac.ir, Lotfi_ab@modares.ac.ir. Tel: 0098-21-82883594. Fax: 0098-21-82883594.

Stem Cells (BMSCs) *in vitro* and *in vivo* (Kang et al., 2003). Because adipose tissue is ubiquitous and easily available in large quantities, it may provide an optional origin of stem cells for mesenchymal tissue re-formation and engineering. In this research, a series of itemized protocols for isolation, characterization, development and differentiation of rat ADSCs into osteogenic, adipogenic as well as, hepatocyte has been investigated.

MATERIALS AND METHODS

Chemicals and reagents:

Dulbecco's Modified Eagles Media (DMEM), fetal bovine serum (FBS), penicillin- streptomycin, phosphate-buffered saline (PBS), 0.25% EDTA solution was obtained from Gibco Bio Cult (Paisley, Scotland, UK). Stem Span media were purchased from Stem Cell Technology (USA). Antibodies for the flow cytometric assay, mouse anti-human monoclonal antibodies for albumin and αFP and the goat anti-mouse FITC-conjugated immunoglobulin G (IgG) were obtained from DAKO (Denmark) and Oxford Biomedical Research, Inc (UK). L-glutamine, Hepatocyte growth factor, dexamethasone, oncostatin M, Alizarin and Oil red staining kits and other reagents were purchased from Sigma Aldrich Co (USA).

ADSCs isolation

Adipose tissue was washed extensively with sterile phosphate-buffered saline to remove cell debris and blood. The extracellular matrix was digested with 0.075% collagenase I at 37°C for 30 minutes to release the cellular fraction. Collagenase I was inactivated with an equal volume of DMEM containing 10% FBS. The infranatant was centrifuged at 250 g for 10 min in order to obtain a high density cell pellet. The pellet was resuspended in proliferation medium consisting of low glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 µg of streptomycin, at 37°C, 5% CO_2, and humidified atmosphere containing 95% air. After 3 days, the nonadherent cell fraction was removed by washing with PBS. These cells were maintained in control medium, until they reached 70 to 90% confluence. Cells were passaged 4 times prior to further analysis in order to ensure removal of contaminating hematopoietic cells (Zuk et al., 2001).

Molecular characterization of ADSCs

Flow cytometric analysis of cell surface markers was performed on cultured ADSCs. To characterize the ADSCs surface expression of the typical marker proteins, approximately 20×10^5 cells were incubated with fluorescent isothiocyanate (FITC) and phycoerythrin labeled antibodies for CD_{90}, CD_{29} and $CD_{45}, CD_{31}, CD_{11}$ and CD_{34}. Isotype-matched irrelevant monoclonal antibodies (mAbs) were used as negative controls. For cell-surface staining, cells were incubated in darkness for 30 min at 4°C in PBS supplemented with 1% BSA. After washing, the cells were resuspended in PBS and measured by using a Coulter Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the results were analyzed with the Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

Storage of ADSCs

ADSCs should be harvested at 80% confluence for freezing. To

collect cells, the culture medium was washed with a small volume of sterile, warm PBS. The cells were treated with trypsin-EDTA solution at 37°C for at least 5 min, or until approximately 90% of the cells have detached from the bottom of the dish. Progress can be monitored under a microscope and the treated cells rounded and floated. An equal volume of stromal medium was added to inactivate the trypsin and the suspension was transferred to a conical centrifuge tube. After centrifuging at 1000 rpm for 5 min, the cell pellet was resuspended in 1 to 2 ml of room temperature cryopreservation medium (80% fetal bovine serum, 10% dimethyl sulfoxide and 10%DMEM). 1 to 2 million viable cells was counted and suspension was aliquot into labeled cryovials, 1 ml/vial. Then the vials placed in an alcohol freezing container, and stored at -70°C overnight. The next day, the frozen vials were transferred to a liquid nitrogen container for long-term storage. Vials of cells should be always stored in the vapor phase of the LN_2 tank (Bruce et al., 2008).

Adipogenic differentiation of ADSCs

3×10^3 ADSCs were plated in 4-well culture plates. The cultured cells were incubated in the adipogenic medium containing DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 µg of streptomycin and treated with 1.7 µM insulin, 1 µM dexamethasone, 200 µM indomethacin and 500 µM isobutylmethylxathine for 12 days. It has been shown that ADSCs in this conditioned medium express, several adipocytic genes including lipoprotein lipase, leptin, GLUT4 develop lipid laden intracellular vacuoles, the definitive marker of adipogenesis (Brian et al., 2005). Fat droplets in adipocytes were derived from ADSCs observed using the oil red O-staining method (Mizuno, 2009). Cell monolayer was fixed in 10% (v/v) formaldehyde solution in aqueous phosphate buffer, washed in 60% isoporpanol and stained with a 0.6% (w/v) oil red O-solution for 10 min at room temperature. This was followed by extensive washing with distilled water prior to destaining in 100% (v/v) isoporpanol for 15 min.

Osteogenic differentiation of ADSCs

For inducing osteogenesis, ADSCs were incubated at 3×10^3 cells/cm^2 in an osteogenic medium containing modified minimum essential medium, 10% FBS, 50 µM ascorbate-phosphates, 1 µM dexamethasone and 10 µM β-glycerophosphate for 12 days. Medium was changed every three days. Under osteogenic conditions, ADSCs expressed genes and proteins associated with an osteoblasts phenotype, including alkaline phosphatase, type I collagen, osteopontin, osteonectin, osteocalcin and bone sialo protein. To assess osteogenic differentiation, the cells were fixed with 90% methanol for 10 min at room temperature and identified by specific histochemical staining for calcium, using the Alizarin red staining kit. The stained material was examined with phase contrast microscopy (Brian et al., 2005).

Hepatic differentiation protocol

When ADSCs were treated with Hepatocyte growth factor, oncostatin M, and dexamethasone, they have shown that they have the potential to differentiate in to hepatocyte-like phenotype by expressing albumin. Hepatocyte growth factor is a potent mitogen that acts via the Hepatocyte growth factor receptor c-Met, a transmembrane protein with an intracellular tyrosine kinase domain. Hepatocyte growth factor plays an important role in liver regeneration and embryonic development. Oncostatin M is a member of the IL-6 cytokine family which regulates hepatocyte differentiation, increases hepatocytes size and enhances cell
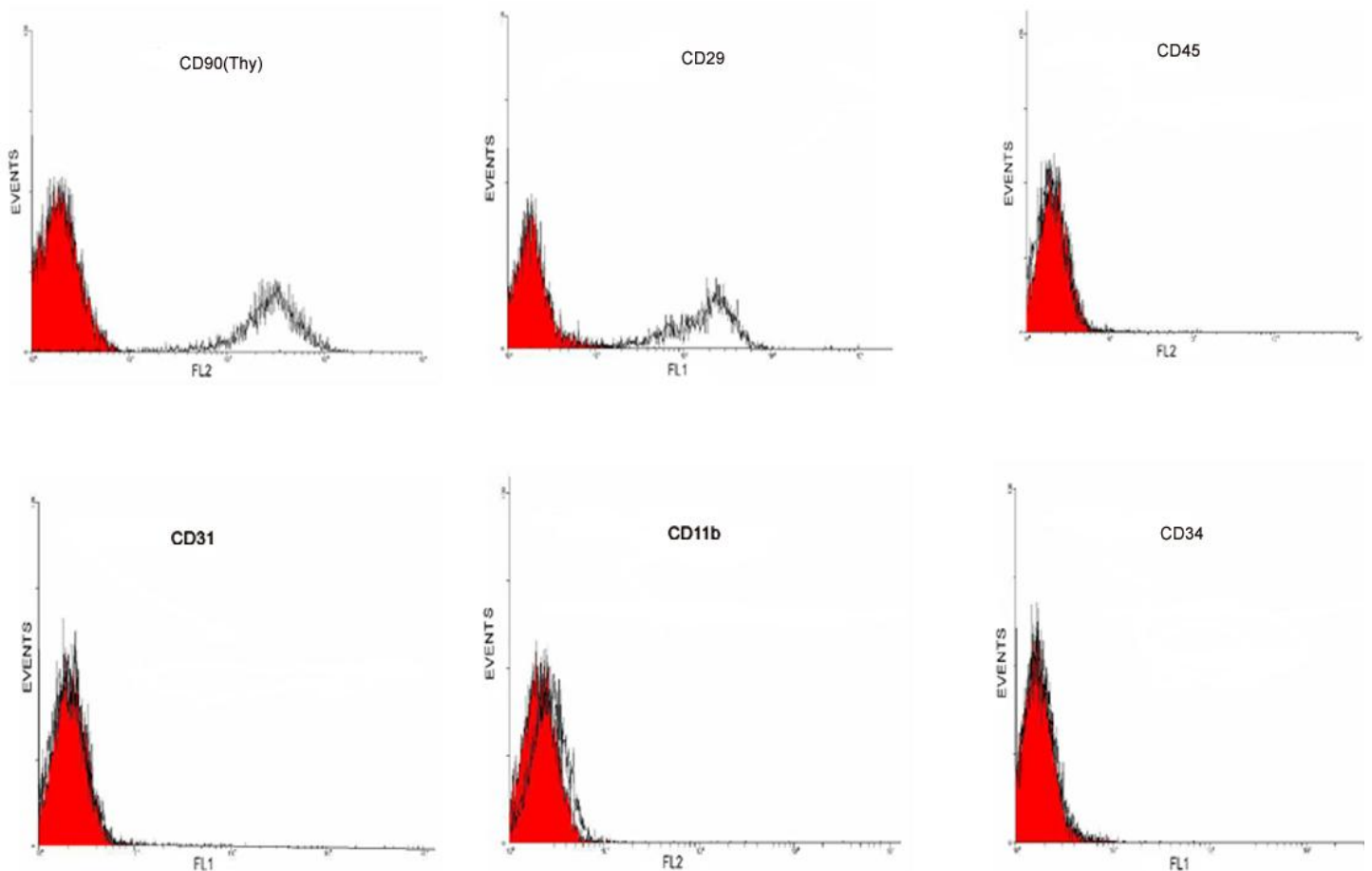
Figure 1. Flow cytometric analysis of the ADSCs. To characterize the ADSCs surface expression of the typical marker proteins, approximately 20×10^5 cells were incubated with fluorescent isothiocyanate (FITC) and phycoerythrin labeled antibodies for CD_{90}, CD_{29} and $CD_{45}, CD_{31}, CD_{11}$ and CD_{34}. Isotype-matched irrelevant monoclonal antibodies (mAbs) were used as negative controls ADSCs were positive for CD_{90}, CD_{29}, and negative for CD_{34}, CD_{45}, CD_{11} and CD_{31}.

differentiation and formation of bile canaliculi (Lazaro et al., 2003). Hepatic differentiation was performed by using a two-steps protocol. Briefly in the first step which lasted for seven days, the cells were cultured in medium consisting of low glucose DMEM supplemented with 15% FBS, 20 ng/Ml of dexamethasone and 10^{-7} mol/L of dexamethasone, followed by 20 ng/mL of oncostatin M for 2 weeks (Stock et al., 2008; Ong et al., 2006).

Fluorescent immunocytochemistry assay

After 3 weeks of cell culture under hepatocyte-conditioned medium, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed with PBS and then permeabilized with 0.4% (v/v) Triton X-100 for 20 min. Corresponding primary antibodies including the mouse anti-human albumin (1:1000) and mouse anti-human αFP (1:500) was then added to the cells and incubated overnight at 4°C. The cells were subsequently washed three times with PBS and incubated with a second fluorescence-labeled antibody and FITC-labeled goat anti-mouse IgG at 3°C, for 3 h in darkness. After washing with PBS the cells were incubated with 4, 6-diamidino-2-phenylindole (DAPI) (1:1000) with the purpose of nuclear staining. The cells were then visualized using a fluorescence microscope (Nikon TE-2000, Japan).

RESULTS

ADSCs isolation

1 g of adipose tissue yields approximately 5×10^3 stem cell, which is 500-fold greater than the number of MSCs in 1 g of bone marrow (Mizuno, 2009). ADSCs Growth rate is significantly higher than bone marrow-derived mesenchymal stem cells.

Characterization of ADSCs

ADSCs were characterized by flow cytometric analysis and differentiation potential upon their exposure to mesenchymal supportive conditions containing adipogenic and osteogenic-specific agents. Flow cytometry analysis revealed that the ADSCs were positive for CD_{90}, CD_{29}, and negative for CD_{34}, CD_{45}, CD_{11} and CD_{31} (Figure 1). To examine whether ADSCs
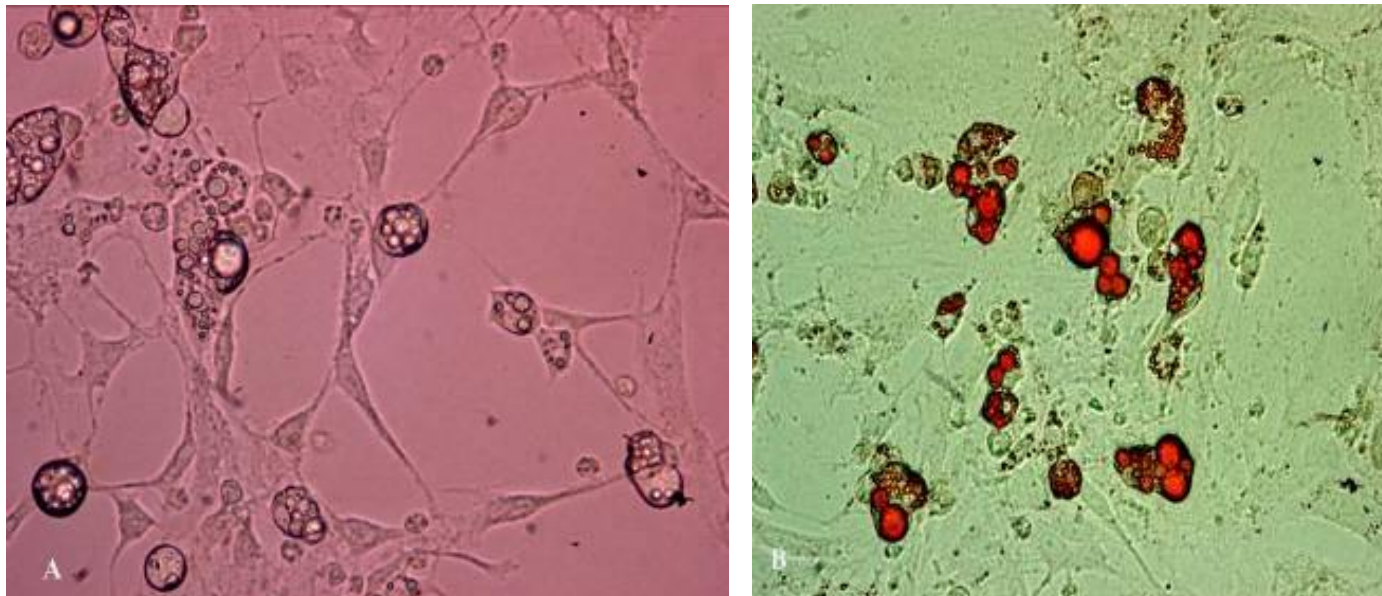
Figure 2. Differentiation of ADSCs into adipocytes (A) Non staining and (B) oil red-staining for adipogenic differentiation.
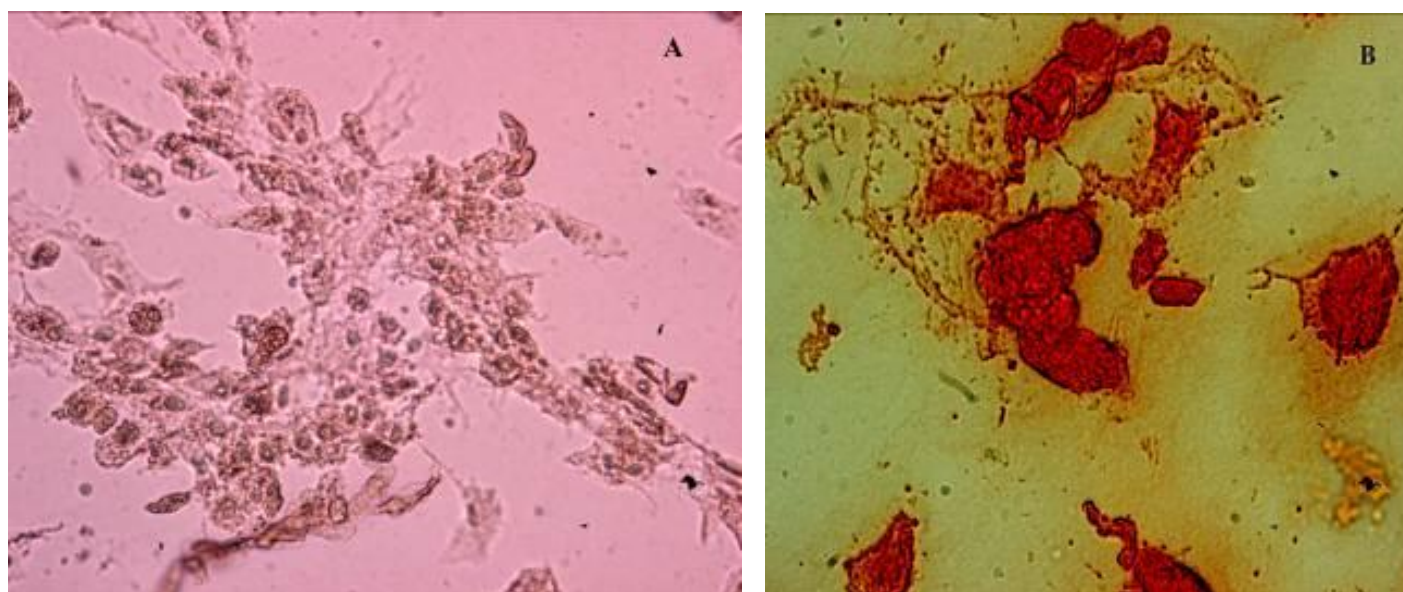

Figure 3. Differentiation of ADSCs into osteoblasts (A) Non Staining and (B) Alzarian red staining for osteogenic differentiation .

have the potential to differentiate into adipocytes, oil red O-staining was performed after 12 days of culturing in adipogenic medium. Fat droplets were seen in red on the surface of cells (Figure 2). We further examined whether ADSCs can differentiate into osteoblasts by Alizarin red staining assay. Presence of calcium deposits, characteristic of osteogenic cells, in differentiated cells after 12 days of culturing in osteogenic medium were observed (Figure 3). ADSCs cultured in normal medium served as a control and were negative for both staining.

Hepatic differentiation of ADSCs

During the initiation step of hepatic differentiation, the cells showed a remarkable transition from bipolar fibroblast-like morphology to a round epithelial-like shape (Yamamoto, 2003) (Figure 4). The contraction of the cytoplasm progressed further during maturation, and most of the treated cells became quite dense and round with clear or double nuclei in the late stage of differentiation (Banes et al., 2007).
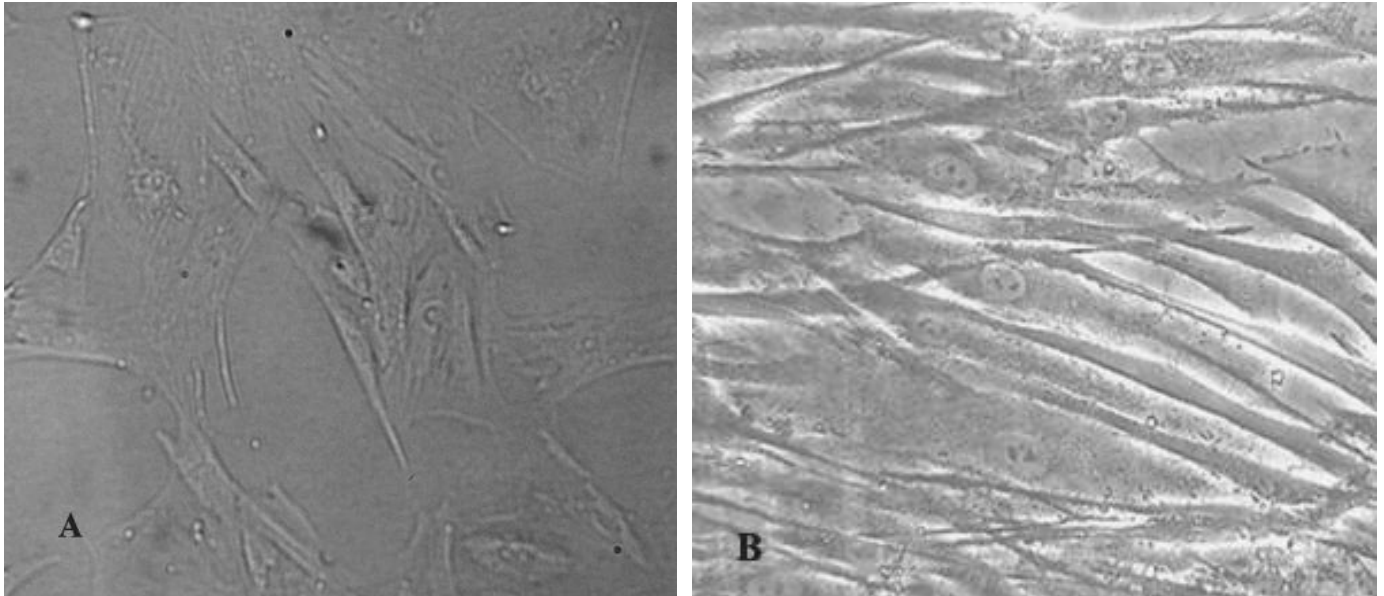
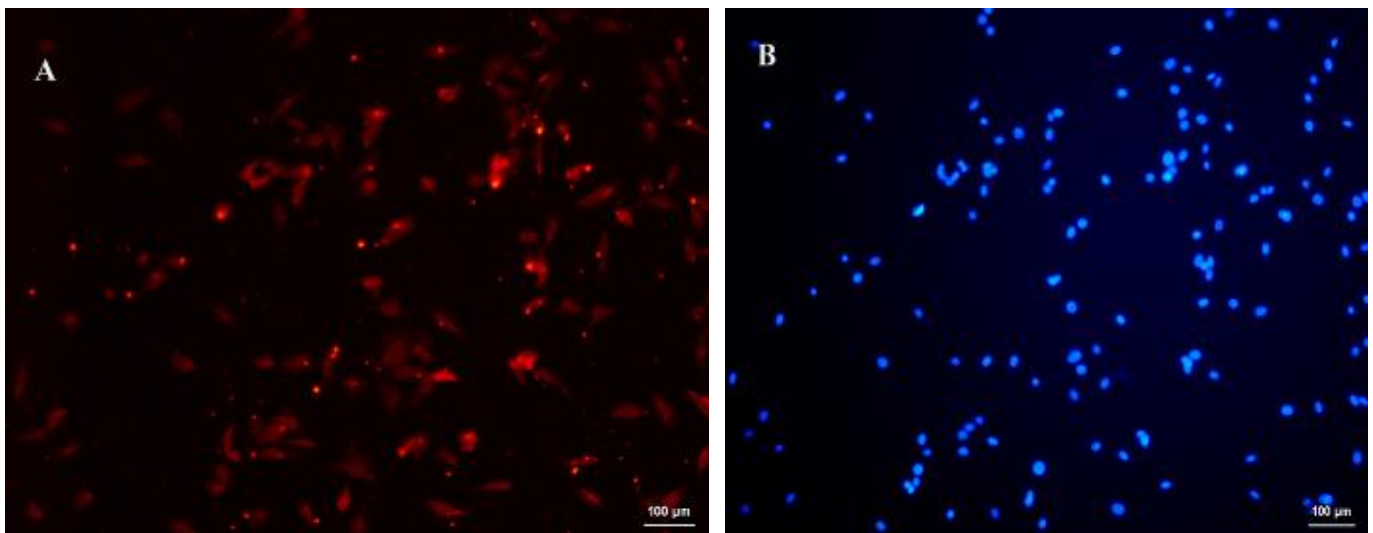**Figure 4.** Differentiation of ADSCs (A) into hepatocyte like cells (B).

Figure 5. Immunofluorescent staining of albumin (A) in differentiated cells; (B) Nuclei were stained with DAPI for Differentiated cells. Scale bar for figures 100 µm.

Immunocytochemical staining

To determine *in vitro* hepatic differentiation of ADSCs, the expressions of albumin (liver specific protein) and αFP (a protein indicative of hepatocyte morphology) were examined. The differentiated cells were positively stained for albumin and αFP on day 21 (Figures 5 and 6). The percentage of albumin and αFP positive cells was 79.23 ± 2.95 and 69.37 ± 3.79 in the differentiated cells. After washing with PBS, cells were incubated with DAPI (4, 6-diamidino-2-phenylindole; 1:1000) for nuclear staining.

The cells were visualized and photomicrographed using a fluorescence microscope.

DISCUSSION

Gimble et al. (2007) has shown that ADSCs deliver to disordered tissues may secrete cytokines and growth factors that stimulate recovery in a paracrine manner (Gimble, 2003). ADSCs modulate the stem cell niche of the host by stimulating the recruitment of endogenous
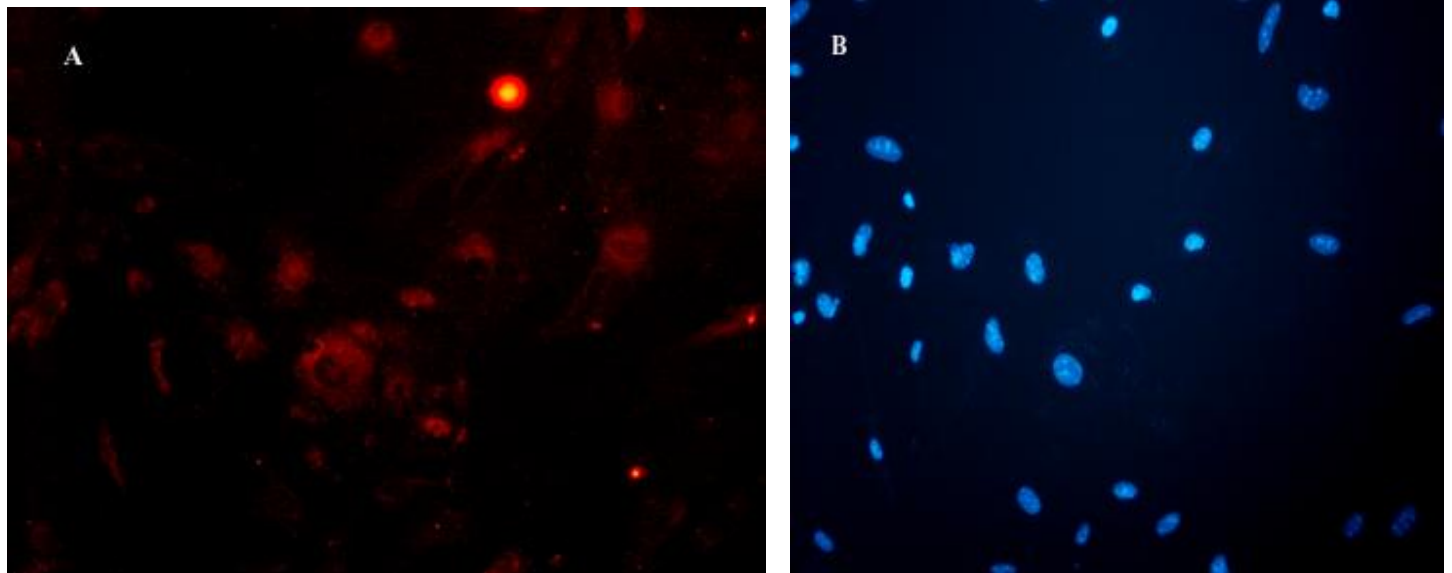
Figure 6. α-fetoprotein staining by immunocytochemistry. (A) In differentiated cells, respectively. (B) Nuclei were stained with DAPI. Scale bar for figures 100 µm.

stem cells to the site and promoting their differentiation through the required lineage path. In a similar way, ADSCs could provide antioxidants, free radical scavengers, and chaperone at an ischemic site. As a result, distributed toxic substances into the local environment would be removed by means of that promoting recovery of the surviving cells (Gimbe et al., 2007). These cells can be also infected by oncoretroviral, adenoviral, and lentiviral vectors mildly to excessive efficiency (Brian et al., 2005). Adipose tissue, like bone marrow, contains a population of stem cells that has extensive self renewal capacity and the ability to differentiate along multiple lineages. These sorts of cells can be acquired in large numbers at high frequency from a tissue origin which can be squeezed in large quantities with least morbidity unlike bone marrow. Thus, adipose tissue represents a potential which is a useful source of cells clinically for cellular therapy, tissue engineering and gene transfer applications. ADSCs supply unique opportunities for investigating strange treatments for an arrangement of inherited and acquired diseases. In addition, ADSCs may supply an opportunity to recognize new molecular targets for drug discovery. In comparison with bone marrow-derived mesenchymal stem cells (BMSCs), ADSCs have the same potential to differentiate into diverse cells and tissues of mesodermal origin, such as adipocytes, cartilage, bone and skeletal muscle and immunocytochemical analysis displayed that ADSCs express albumin and α-fetoprotein during hepatic differentiation. Based on this progress, several clinical implications for cell therapy and tissue engineering are generously favorable (Majumdar, 1998).The easily repeatable access to adipose tissue provides a clear advantage for the isolation of MSCs, and both the isolation and culture techniques are easier to perform than bone marrow isolation. However, as mentioned earlier, adipose tissue is now regarded as an affluent source of stem cells.

Conclusion

In summary, adipose tissue like bone marrow contains a population of cells that has extensive self renewal capacity and the ability to differentiate along multiple lineages. The cells possessing this activity can be obtained in large numbers at high frequency from a tissue source that can be extracted in large quantities with minimal morbidity, unlike bone marrow.

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