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Growth kinetic comparison of Human Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue and Decidua

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ABSTRACT

Introduction: Mesenchymal stromal cells (MSCs) are undifferentiated cells with high proliferation potency. These cells can be characterized by their ability to self-renew and differentiate into specialized cell lineages. Therefore, these cells are considered as an attractive source of stem cells for cell therapy. However, MSCs are slightly different from different sources. The most used stem cell

types, in both human and veterinary fields, are MSC derived from bone marrow and adipose tissue. Nowadays, there is a great interest in using stem cells derived from fetal tissues, such as decidua, which can be obtained non-invasively at delivery time and are an unlimited source of stromal cells. *Materials and methods:* In this study, biological characteristics of stromal cells from adipose tissue (AT-MSC), bone marrow (BM_MSCs), and decidua (DSC), such as proliferation capacity, passage capacity, colony formation and cell viability after cryopreservation were compared. *Results:* Based on our results, population doubling time of DSC in comparison with BM_MSC and AT-MSC is shorter (under 20 hours to passage 10), and these cells possess the highest potential in colony formation and ability of self-renewal. Also, these cells were well-maintained their viability after Cryopreservation up to 80%. *Conclusion:* DSCs have higher colony-forming ability and long-time passage capacity. Due to the significant proliferation capacity and great viability after cryopreservation, DSCs may be considered as a new source of stromal cells for application in cell-based therapies.

Keywords: Mesenchymal stem cell, Decidua, Cryopreservation, Proliferation capacity, Colony formation.

1. INTRODUCTION

About half a century ago Alexander Friedenstein introduced a type of cells from bone marrow that was Non-hematopoietic and called them 'mesenchymal' precursor cells (Zhao et al., 2016). These cells communicate through cell contacts, growth factors, cytokines, and extracellular matrix proteins, creating microdomains or niches and regulating their self-renewal, differentiation and quiescence. So, these cells were considered by clinicians and medical researchers. In vitro studies of these cells showed that they express special surface markers that referred to their proprietary features such as adherence, migration, and immunomodulation of the immune system (Sasaki & Honmou, 2017). Over the years, it has become increasingly clear that such cells can differentiate in vitro into a diversity of mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes (Alabdulkarim et al., 2017). These cells can also be isolated from other organs and tissues such as adipose tissue (Pendleton et al., 2013), amniotic fluid (Vanishree et al., 2016), placenta (Chen, 2017), umbilical cord (Campos, 2014), and peripheral blood (Heo et al., 2016). Because of their properties, these cells are a good candidate for accelerating the wound healing, tissue regeneration and immunosuppression. One of the limitations of using these cells is decreasing the growth rate of cells with prolonged cell culture (Chen et al., 2006). It means that the rate of cell proliferation is reduced. The cost-effective production of human mesenchymal stromal cells (hMSCs) for off-the-shelf and patient specific therapies will require an increased focus on improving product yield and driving manufacturing consistency. Therefore, the aim of this study was to assess the comparative rate of mesenchymal stromal cells from decidua with bone marrow and adipose tissue as a golden standard and accessibility source respectively.

2. MATERIAL AND METHODS

Ethical Approval

The study protocol was approved by the Ethics Committee of the Motamed Cancer Institute (ethical code: IR.ACECR.IBCRC.REC.1396.19).

Cell preparation and cell culture

Ethical approval was delivered by the institutional ethics committee. AT-MSCs and BM-MSCs prepared from Iranian Biological Resource Center. Briefly, BM-MSCs were isolated from BM aspiration of healthy donors. Approximately 12 ml BM aspirate were diluted by adding DMEM (Gibco, USA) supplemented with 10% FBS (SFB, Gibco, USA). After preparing a single-cell suspension, the BM-MSCs cultured at a density of 5×10^3 cell/cm^2 in T-75 flasks. After 3 days, non-adherent cells were removed and culture of adherent cells continued. When confluency of cells reached 70-80%, cells were detached and passaged to more flasks.

AT-MSCs were obtained from human lipoaspirates. Lipoaspirates were washed with PBS and then treated with 0.075% collagenase type 4 (Sigma-Aldrich, UK) for 30 min at 37°C. After homogenization, collagenase was inactivated with DMEM supplemented with 10% FBS and were centrifuged for 10 min at 1200 g for pellet obtaining. The pellet was filtered with 100-µm mesh filter to remove cellular debris and incubated at 37°C/ 5% CO2 in DMEM, 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin). After 3 days, non-adherent cells were removed and culture of adherent cells continued. When confluence of cells reached 70-80%, cells were detached and passaged to more flasks.

Decidua-derived mesenchymal stem cells

Full term human placentas were retrieved from healthy women who underwent elective cesarean section. Afterwards, decidua basalis was dissected from placenta, chopped into small pieces (1-2 mm3) and transferred into the 10 cm2 plates containing DMEM medium supplemented with 10% FBS plus100 U/mL penicillin and 100 µg/mL streptomycin. Then the plates were incubated in humidified air containing 5% CO2 at 37 °C and were kept undisturbed for 10 days, allowing cell migration from the margins of explants, the procedure referred as explants method.

Cells were cultured in 75cm2 plastic flasks (Sarstedt, USA) contained culture medium consisting of DMEM low glucose (Gibco, USA), supplemented with 10% fetal bovine serum (SFB, Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) and 1.1% amphotericin B (Gibco, USA) and incubated at 37.5º C in a humidified atmosphere containing 5% CO2. Culture medium was changed every 48 hours until 80% confluence was reached, when adherent cells were detached with trypsin and passaged to subcultures until passage 10.

Flow cytometric analysis

For cytometric analysis, MSCs from different tissues were cultured to 70-80% confluences then cells were harvested with trypsin solution (0.25% trypsin/EDTA) and cells were stained with antibodies against human CD44, CD73, CD90 (FITC, BD Biosciences), HLA-DR ,CD34, CD11b, CD45 (PE, BD Biosciences), and CD105 (APC, BD Biosciences) for 15 min at room temperature. Corresponding mouse isotype antibodies served as controls. The cells were washed with PBS and fixed with 1% (*v/v*) paraformaldehyde. The cells were then analysed using a flow cytometry instrument (ABI, USA) and data analysis software (FlowJo 10.0.7; TreeStar, USA).

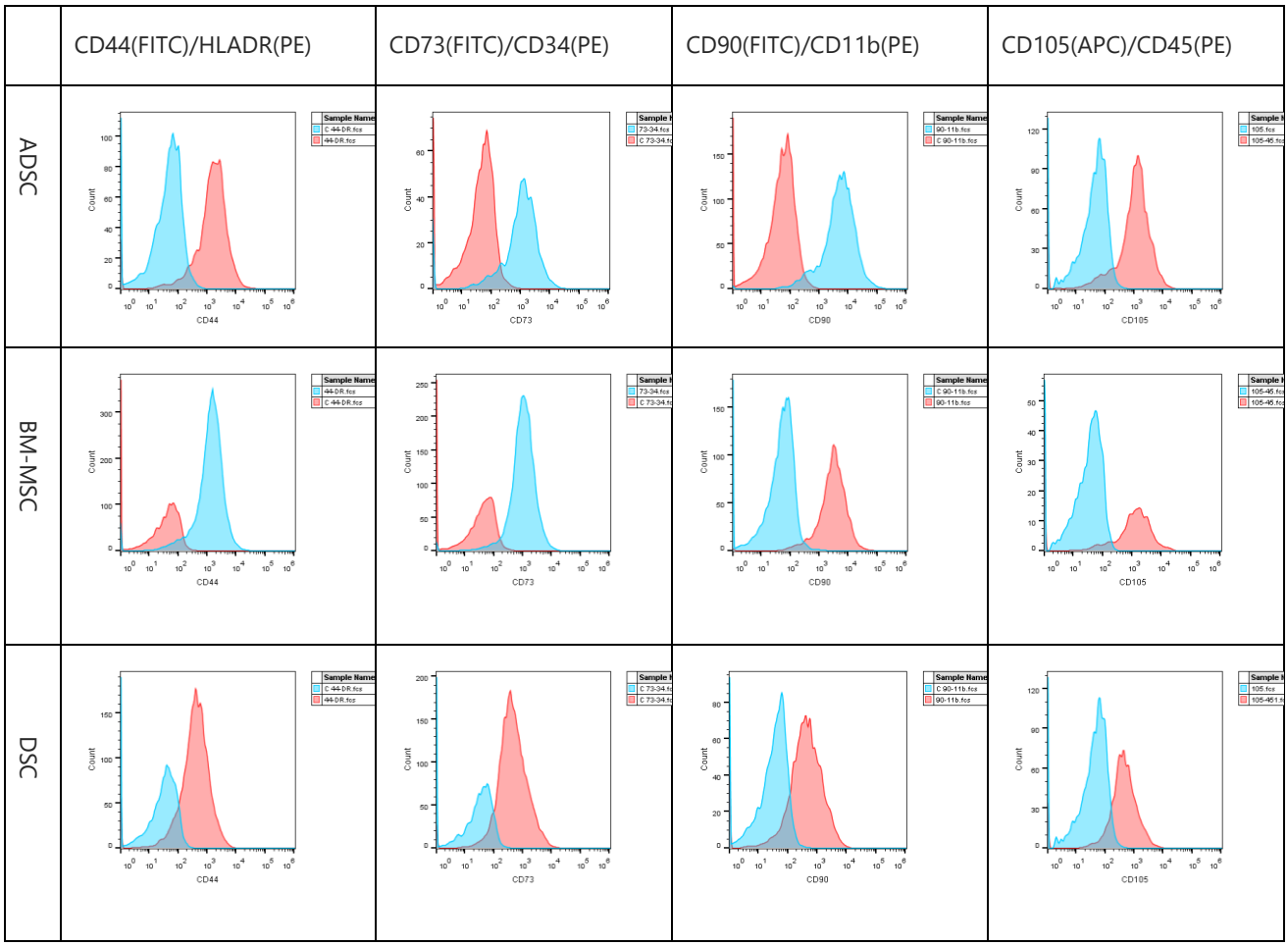

Figure 1 Representative plots of phenotype of BM-MSCs, AT-MSCs and decidual stromal cells (DSCs).

Confirmation of multilineage differentiation of MSC

Adipogenic differentiation was performed by culturing MSC for 3 weeks in adipogenic Medium (10% FBS, 1 mM dexamethasone, 100 µg/mL 3-isobutyl-1-methylxanthine, 10 µg/mL insulin, and 100 U/ml penicillin/streptomycin) and assessed using an Oil Red O stain to indicate intracellular lipid accumulation (10) .for Osteogenic differentiation cells were seeded at 2 × 10^5 cells/well in 6-well plates. When MSCs reached 80-90% confluence, differentiations were induced with osteogenic Medium (10% FBS, 0.1 mM dexamethasone, 10 µM β-glycerophosphate, and 50 µg/ mL ascorbic acid in α-MEM). After 3 weeks of incubation, cells were stained with alizarin red for examining the calcium deposits (10). Chondrogenic Differentiation was performed using the micromass culture technique. Briefly, to pellet cells for chondrogenic differentiation, 2 × 10^5 cells were centrifuged in a 15-ml tube and were incubated overnight at 37.5° C. After 24 h, the media were replaced with chondrogenic differentiation medium (20 ng TGFβ1, 10 ng insulin, 100 nM dexamethasone, and 100 µM ascorbic acid) without disturbing the pellet and cultures were maintained in CM for 3 weeks prior to analysis. Chondrogenesis was confirmed by toluidine blue stain for indicating the presence of glycosaminoglycan and glycoproteins (Jin et al., 2010).

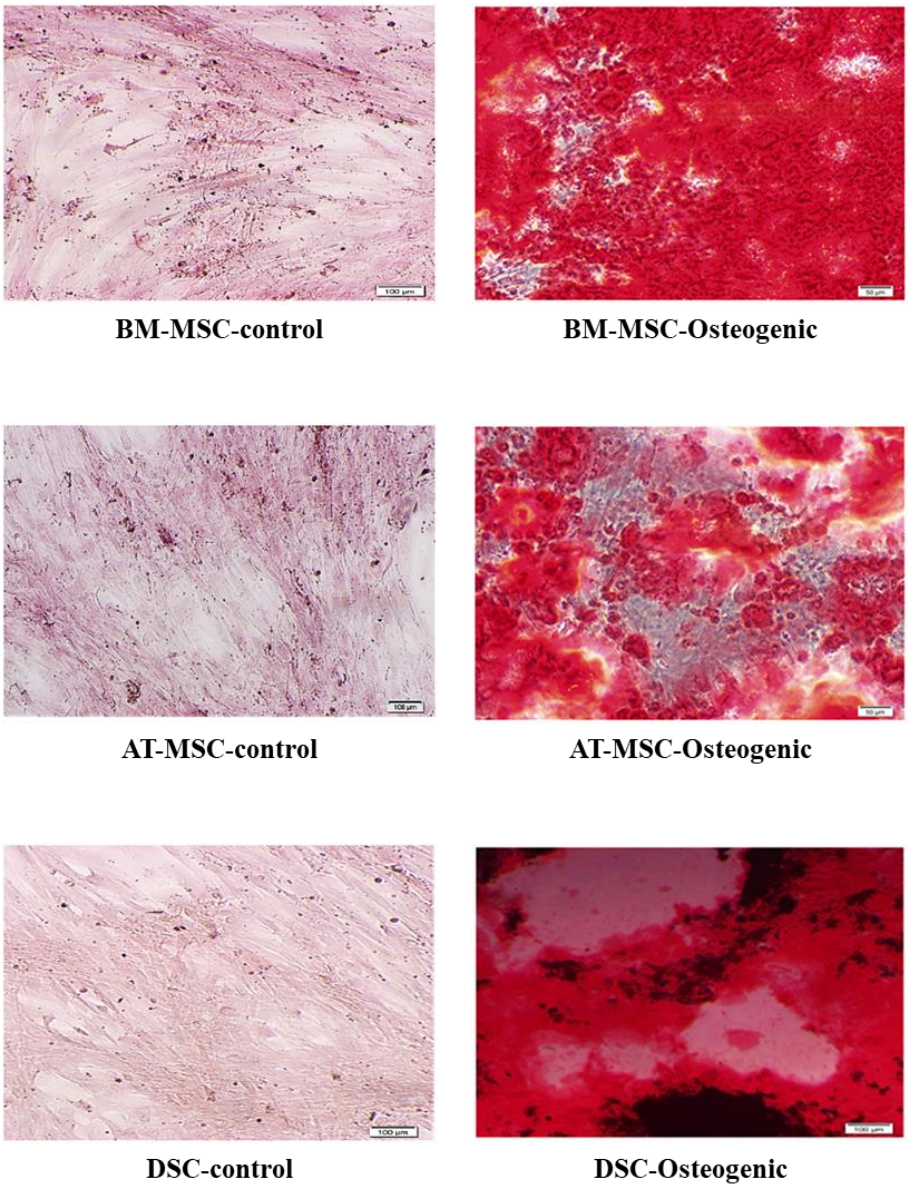

Figure 2 osteoblastic differentiation of bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC)-derived mesenchymal stem cells.

Colony-forming unit-fibroblast (CFU-F) assay

The self-renewal capacity of the cells can be evaluated by CFU-F assay. To assess the self-renewal capacity of the cells, 1×10^3 cells at passage 2, 5 and 10 were seeded in 100-mm plates (Corning Inc., Corning, NY, USA). Following cultivation for 14 days, the cells were washed with phosphate-buffered saline (PBS; Invitrogen) and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. Stained colonies with >50 cells were counted.

Population doubling time (PDT)

The generation time (average time between two cells doublings) for the BM_MSCs, AT-MSCs and DSCs in different passages was calculated for P1 to P10 using the following formula (Figure 1).

PDT=culture time (CT)/ population doubling level (PDL)

The proliferation of MSCs under different passage was assessed using appropriate kinetic for calculation of population doubling (PD) rate and generation time as described by Tanavde et al. (2009). The passaged cells were allowed to reach the confluence of 80%. PDL was also calculated according to the following formula:

PDL= log Ni/Nf×3.31

In which Ni and Nf represent the numbers of initiating and harvesting cells, respectively (Figure 2).

Viability analysis

After P2,P5 and p10, MSC from BM-MSCs, AT-MSCs and DSCs were trypsinised and the pellet was resuspended into 1.5ml cryotubes containing freezing medium (95% FBS supplemented with 5% DMSO). Cryotubes were stored in a controlled cooling container (Mr. Frosty, Nalgene, USA) in -80ºC freezer for 24 hours and then transferred to liquid nitrogen container at -196 C°. After 3 to 6 months, samples were thawed and cell viability was analyzed by trypan blue staining method.

$$x = \frac{\text{number of live cells}}{\text{number of dead cells}} \times 100$$

Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Statistical significance was determined by ANOVA followed by Tukey´s post hoc tests. A $P < 0.05$ was considered statistically significant.

3. RESULT

Morphology

Mesenchymal stem cells obtained from different sources were cultured. After initial culture, mesenchymal stromal cells derived from adipose tissue, bone marrow and decidua tissue were attached to the plastic surface. Adherent fibroblastoid cells were observed after 4-5 days and showed spindle-shaped fibroblastic morphology within passage 2, 5 and 10 and these cells reached 80-90% confluence. However, MSCs from 3 origins were morphologically homogeneous, small and spindle-shaped, but AT-MSCs and BM-MSCs with increasing of passage number gradually lost their shape and expanded while DSCs retained their form. There were no differences in terms of time for adhesion and fibroblastoid morphology between BM-MSC, AT-MSC and DSC (Figure 3).

Immuno profile of isolated cells

Cells isolated from adipose, bone marrow and decidua tissues were investigated for the expression of cell-specific markers. Stromal cells form all of sources, displayed positive expression of MSCs markers including CD44, CD73, CD90, and CD105. But these were negative for HLA-DR and hematopoietic specific markers such as CD45, CD34 and CD11b according to ISCT criteria (Figure 2).

Multilineage differentiation potential

To examine the multilineage capacity of stromal cells, cells isolated from 3 sources, were differentiated toward osteogenic, adipogenic and chondrogenic lineages. The three-lineage potential is a standard for defining stromal cells. Multilineage potential in all MSCs was assessed by specific staining for each lineage. Adipogenesis was tested by staining of lipid droplets with oil Red O, osteogenesis by ALP staining and chondrogenesis by toluidine blue staining (Figure 1,4,5). These results indicate that MSCs from 3 sources can differentiate into mesodermal lineage including osteoblasts, chondrocytes and adipocytes.

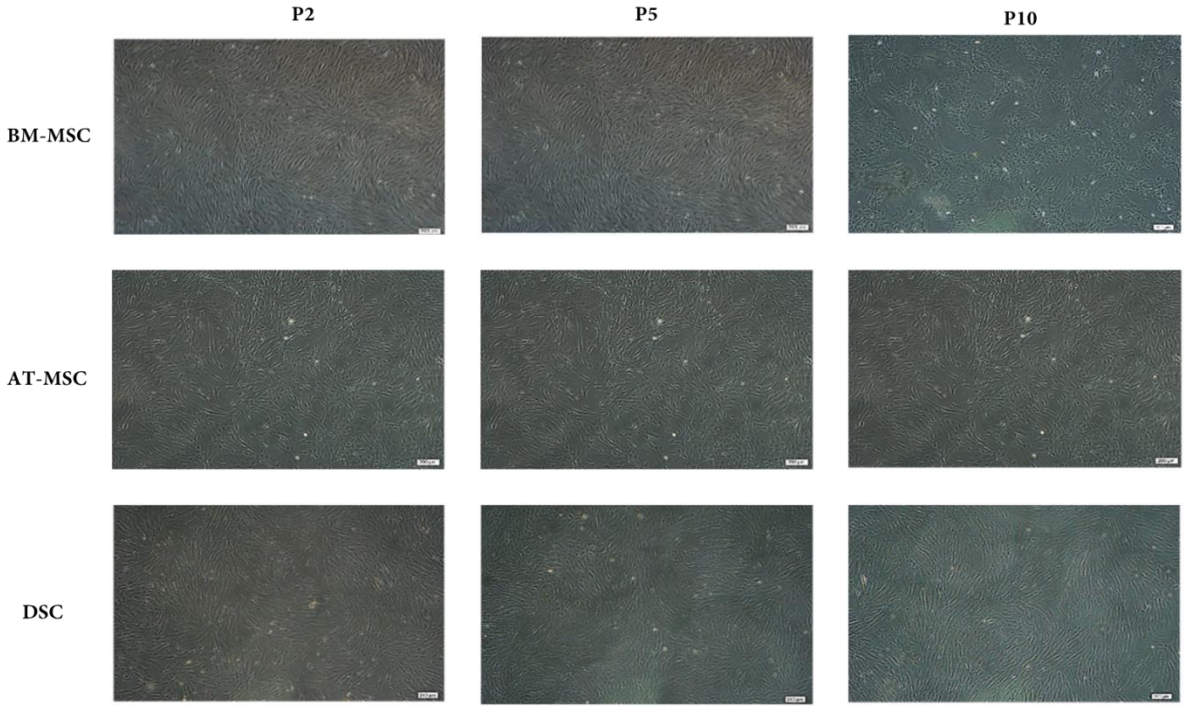

Figure 3 Morphology of bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC)-derived mesenchymal stem cells. Scale bar = 100 µm.

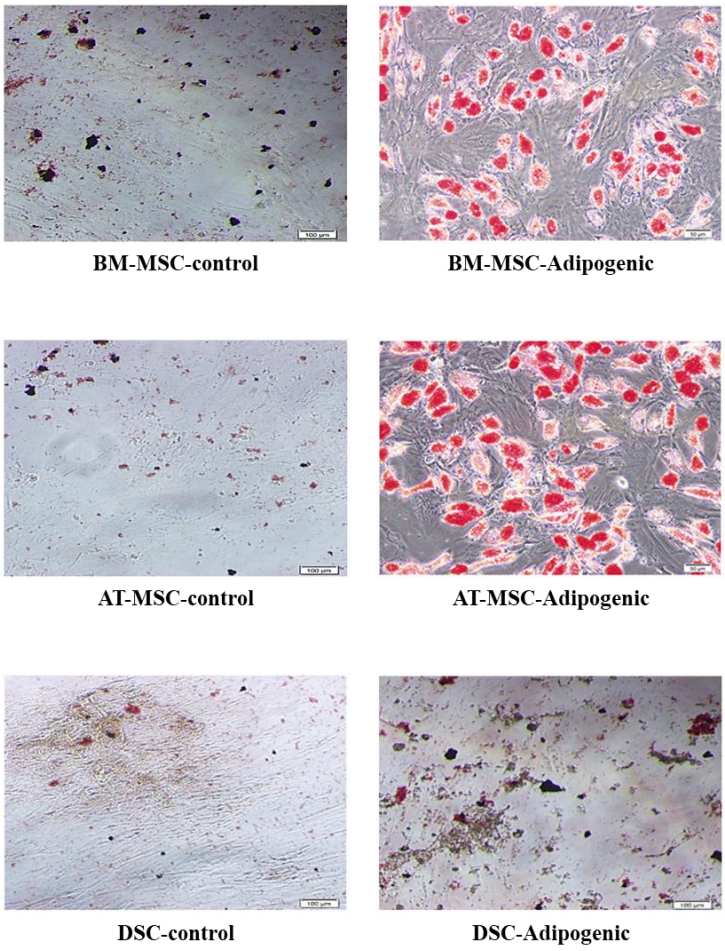

Figure 4 Adipogenic differentiation of bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC)-derived mesenchymal stem cells.

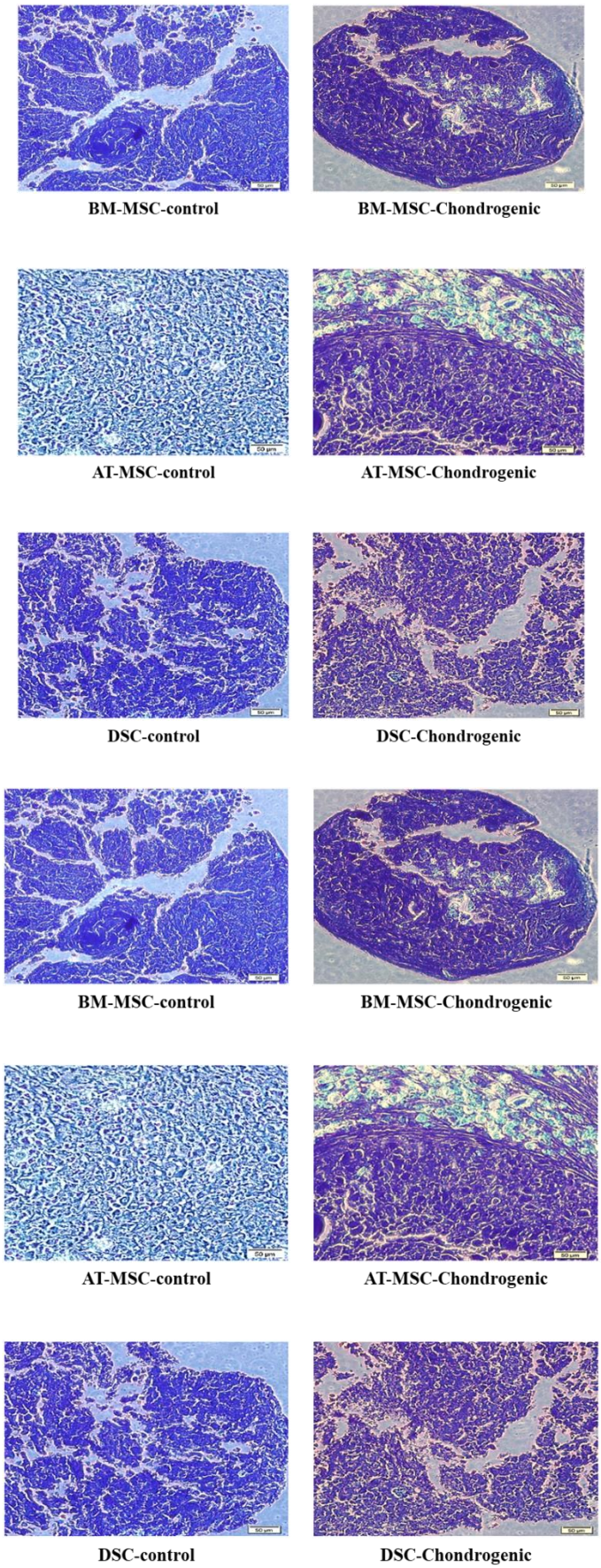

Figure 5 Chondrogenic differentiation of bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC)-derived mesenchymal stem cells.

Growth characteristics of MSCs derived from different tissues

Self-renewal capacity: The CFU-F assay was used to examine the self-renewal capacity of the cells. Although the mesenchymal stromal cells derived from decidua tissue exhibited better growth characteristics than the other cells, There were also a significant difference in the number of CFU-Fs following cell seeding at 1×103 cells in 100-mm plates after 14 days (Figure. 6). The DSCs displayed a higher self-renewal capacity regardless of growth rate ($p<0.05$). According to our results, clonogenicity capacity of MSCs derived all tissues was retained to passage 2. However, clonogenic ability of AT-MSCs and BM-MSCs gradually decreased with increasing of passage numbers and age of cells but DSCs could retain this capacity to passage 10 greatly.

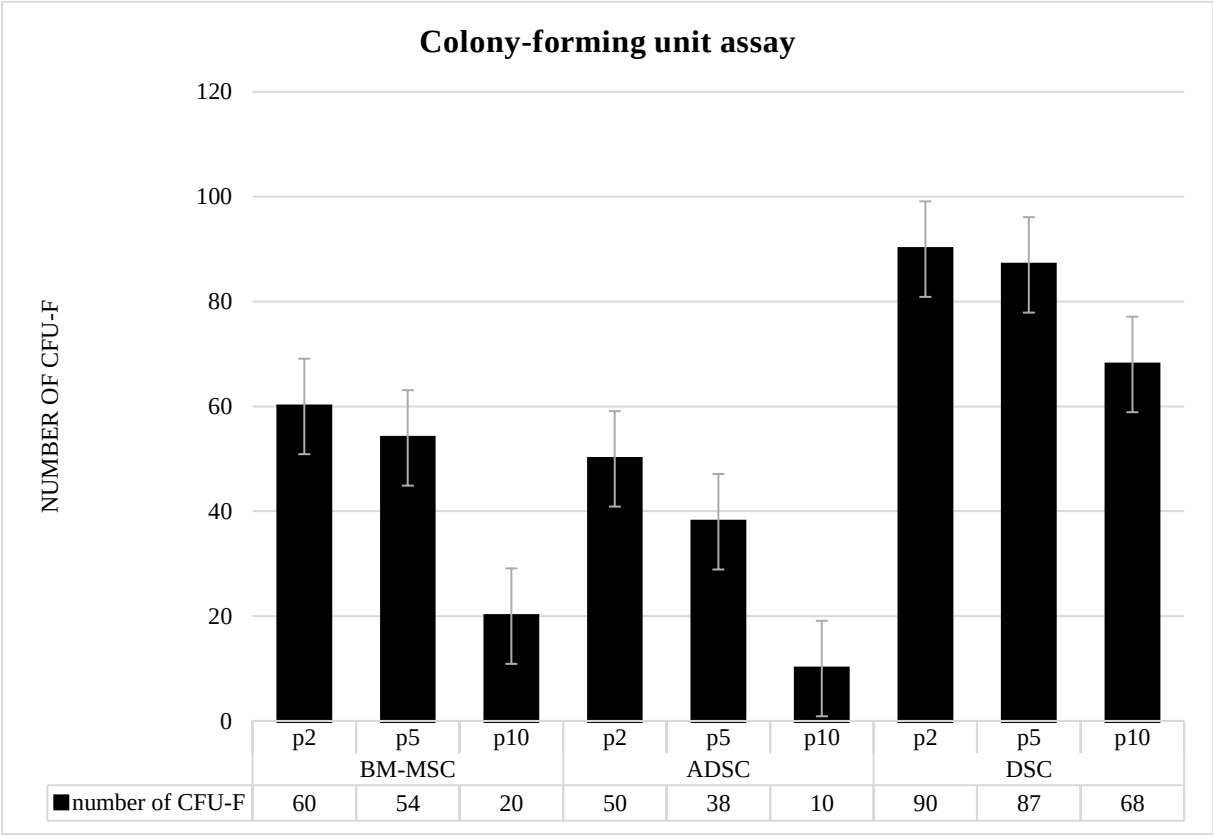

Figure 6 Clonogenic capacity was measured by colony forming unit-fibroblast (CFU-F) assay. **p<0.01.

Cell proliferation

As depicted in figure 2, DSCs possessed significantly shorter population doubling time compared to BM-MSCs and AT-MSCs during P1 to P10 ($p<0.05$). Mean population doubling time for DSC P1 cells was approximately 18 h and remained almost intact until the tenth passage. DSC could expand every 3 days for 10 passages without any significant changes in either the morphology or growth rate, representing the high proliferation capacity of these cells. However, the population doubling time for BM-MSCs and AT-MSCs were 35 and 40h respectively and begin to increase markedly after the sixth passage. Therefore, DSC is the highest proliferative MSC among others (Figure 7,8).

Viability assay

MSC from all samples were cryopreserved after P3 and thawed subsequently for viability. All DSCs samples showed above 80% of viability post cryopreservation, but in the AT-MSC and BM-MSC viability of the cells after cryopreservation reduced to 70% respectively in passage 10 and 9 (Figure 9).

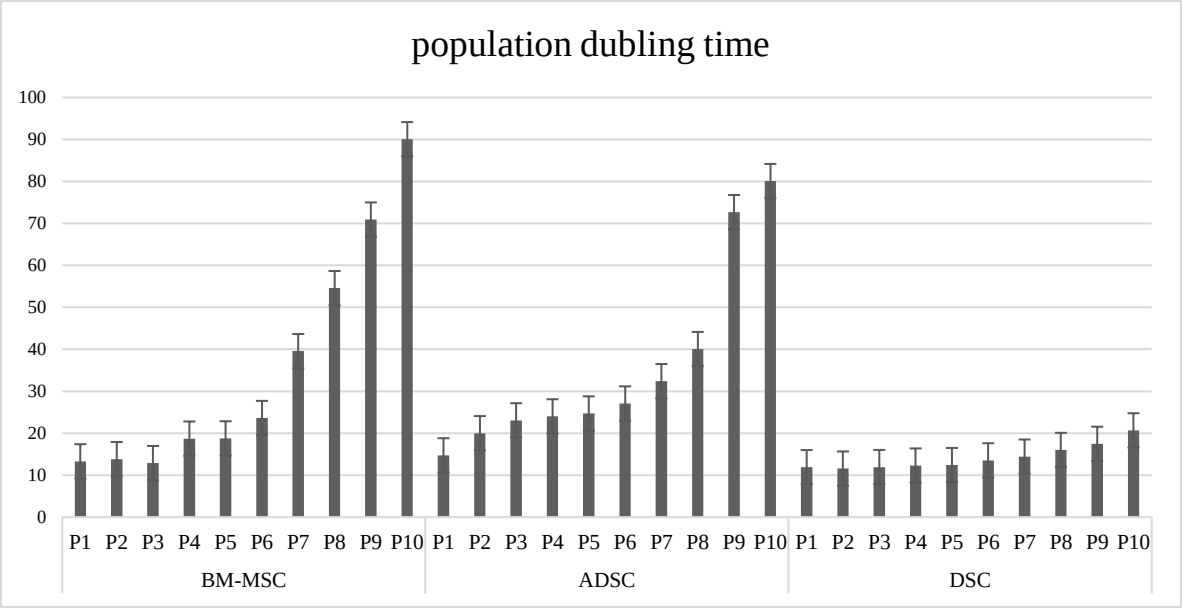

Figure 7 Population doubling time (PDT, measured in hours) was determined at each sub cultivation. MSCs derived from bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC). $^{**}p<0.01$.

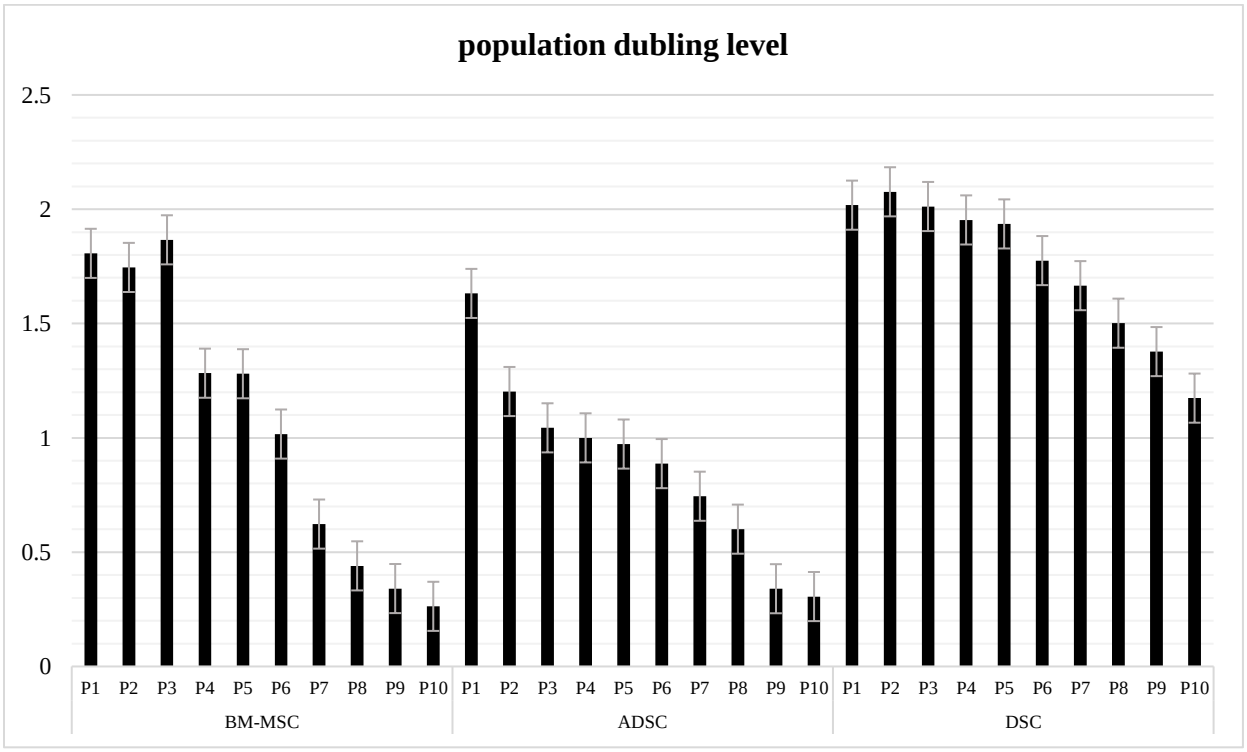

Figure 8 The population doubling level, defined as the total number of serial cell passaging before reaching replicative senescence. $^{**}p<0.01$.

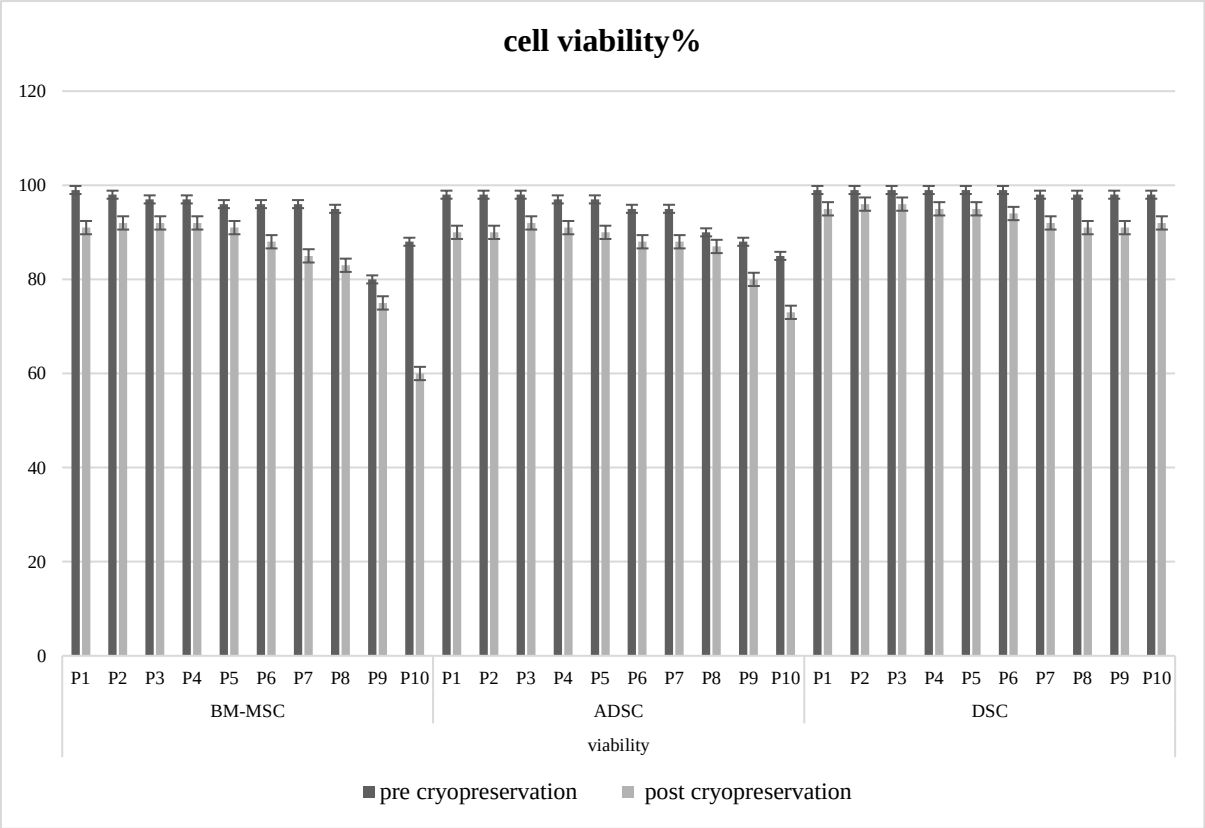

Figure 9 Assessment of viability of MSCs derived from bone marrow- (BM-MSCs), adipose tissue (AT-MSCs) and decidua (DSC). $^{**}p<0.01$.

4. DISCUSSION

Mesenchymal stromal cells with self-renewal and multi-lineage differentiation capacity, are an attractive source of stem cells for cell therapy approaches (Jiang et al., 2002; Caplan, 2007). Although BM-MSC and AT-MSC are the primary source of MSCs for clinical use, because of harvesting by invasive procedures are limited (Mosna et al., 2010; Alves et al., 2012). Therefore, in order to establish a clinical therapy, it is of importance to be able to obtain the cells of interest easily, and to be able to expand them to numbers sufficient for efficient clinical therapy. Researchers are therefore investigating suitable alternative cell sources. Decidua-derived MSCs have attracted much attention and are considered most suitable because can be isolated through non-invasive methods easily. Studies has indicated that stromal cells from the placenta could be isolated and that the cells had a comparatively great expansion potential (in't Anker et al., 2004). DSCs possess characteristics similar to BM-MSCs and AT-MSCs such as spindle-shaped morphology, differentiation potential and surface markers that are defined by ISCT criteria for MSCs (Bosch et al., 2011; Dominici et al., 2006).

However, studies lack comparative data that indicate kinetic differences between MSCs of these various sources. So, a major concern that remains is to determine which cell source is most appropriate. In our report, we compared DSCs to BM- and AT-MSCs, which exhibit typical MSCs characteristics regarding to their growth kinetic. Stromal cells from 3 origins, display the principal characteristics of MSCs (Dominici et al., 2006). MSCs derived from all three sources produce spindle cells *in vitro*(Körbling & Estrov, 2003). However, differences among the cells from various tissues became evident during consecutive cell culture. 1) There are obvious differences in cell–cell contact inhibition; AT-MSCs and BM-MSC are significantly inhibited while DSCs are not. 2) AT-MSC has the largest cell volume, and DSC the smallest. 3) Different numbers of MSCs are obtained from different sources (Pittenger et al., 1999). Here we also compare the potentials of proliferation, self-renewal and viability of BM-, AT- and placenta MSCs. Although MSCs could be isolated successfully from the three different tissues, the resulting cells showed noticeable differences in cultivation rates.

Immunophenotypic analysis results showed that cells from all sources represent similar expression of surface markers with consistent MSC profile including strong expression of CD44, CD73, CD90, and CD105. However, CD34 and CD45 were not expressed. It has been confirmed that placenta-derived MSCs have persistently high proliferation rates up to passage 20 (Semenov et al., 2010). All MSCs of the present study also maintained fibroblast-like shape until passage 10 but BM- and AT-MSCs gradually lost their spindle morphology and became wider in comparison with DSCs that could retain their morphology. In addition, all MSCs showed multi-lineage differentiation potentials. MSCs from different tissues demonstrated different capabilities in terms of cell proliferation. Growth curve analysis and passage experiments indicated that DSCs had the greatest expantion potential, followed by BM-MSCs and AT-MSCs. DSCs could be cultured for longer periods and displayed the highest expantion capacity, whereas AT-MSCs had the lowest growth rate (PDT=40h). DSC showed significantly higher passage ability than BM-MSCs and AT-MSCs.

Our study showed all three kinds of MSCs had colony-forming abilities but DSCs showed more colonies in CFU-F assay in compared to those from adipose and bone marrow tissues in passage 2. As the passage number increased, AT- and BM-MSCs lost their clonogenic ability gradually but DSCs could maintain their stemness after long-term culture. An important part of cell therapy is the establishment of cell banks that allows greater resource availability for the treatment of diseases (Perry et al., 2008). Therefore, it is obligatory to operate safe protocols that allow cells to preserve their viability for a long period (Caplan, 2007). In the present study, after thawing, MSC from DSC showed higher cell viability (up to 80%) when stained with trypan blue and in contrast with AT- and BM-MSCs, with increasing the passage number, the reduction of viable cell of DSCs was insignificant.

5. CONCLUSION

Based on the proposed aims, we can conclude that DSC may be easier isolated than Bone marrow and adipose tissue. On the other hand, these cells retain growth kinetic properties such as PDT, PDL and self-renewal over 10 passages. This means that these cells also maintain their proliferative capabilities until passage 10. As the time to reach a certain number of cells is shorter in these cells so we can suggest that the use of these cells is more cost-effective; However, more studies related to the identification of mechanisms and molecules that participate in the biological functions and that control the survival and proliferation of these cells after in vivo application are required.

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Authors' Contribution

This investigation was designed and managed by Dr. behnamSadeghi and Dr.KazemParyvar. The experiments were performed and analyzed by Sepide Kazemi.

Conflict of Interests

The authors declared no conflicts of interest.

Ethical Approval

The study protocol was approved by the Ethics Committee of the Motamed Cancer Institute (ethical code: IR.ACECR.IBCRC.REC.1396.19).

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The study was granted by Dr.Behnam Sadeghi Head of regenerative medicine and immunotherapy department, Motamed Cancer Institute.

Abbreviation

MSC	Mesenchymal Stem Cell
CFU-F	Colony Forming Units-Fibroblastic
GvHD	Graft-versus-Host Disease
UCB	Umbilical Cord Blood
BM	Bone Marrow
AT	Adipose Tissue

DSC Decidua Stromal Cell
HSC Hematopoietic stem Cell
PDL Population Doubling Level
PDT Population Doubling Time

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