

Original Research

Comparative characteristics of mesenchymal stem cells derived from reamer-irrigator-aspirator, iliac crest bone marrow, and adipose tissue

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Abstract: Mesenchymal stem cells (MSCs) have been considered promising tools for new clinical concepts in supporting cellular therapy and regenerative medicine. More recently, Ream/Irrigator/Aspirator (RIA) was introduced as a source of MSCs. In this study we compared MSCs derived from three different sources (iliac crest bone marrow (ICBM), adipose tissue (AT), and (RIA)) regarding the morphology, the success rate of isolating MSCs, colony frequency, expansion potential, osteogenic and chondrogenic differentiation capacity. MSCs were isolated from three different sources and flow cytometric analyses were performed for cell characterization. Colony-forming unit-fibroblast (CFU-F) assay and population doubling time (PDT) were evaluated for MSCs derived from three different sources and differentiation potential of RIA, ICBM-, and AT-MSCs were determined by staining. Additionally, gene expression profiles for tissue specific markers corresponding to osteogenesis and chondrogenesis were analyzed using real time polymerase chain reaction (RT-PCR). Cultured with the appropriate condition, osteogenic and chondrogenic differentiation could be confirmed in all MSC preparations. Flow cytometry analysis indicated that RIA- and AT-derived MSCs have more homogenous populations than ICBM-MSCs. A comparison of the colonogenic ability in different tissues by CFU-F assay after 10 days showed that more colonies are formed from RIA-MSCs than from ICBM-MSCs, and AT-MSCs. AT-MSCs, were dispersed with no obvious colonies. The RIA-MSCs underwent osteogenesis and chondrogenesis at a faster rate than ICBM and AT-MSCs. Direct comparisons of RIA- to ICBM- and AT-MSCs have shown the RIA-MSCs have higher differentiation toward osteoblast and chondrocytes compared to other sources of MSCs. Hence, RIA-MSCs may be recommended as a more suitable source for treating orthopedic disorders.

Key words: Differentiation potential, cell therapy, stem cells, regenerative medicine, orthopedic tissue engineering.

Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells that have been isolated from both adult and fetal tissues (1). In the early 1970s, Friedenstein et al. described the existence of multipotent mesenchymal cells in mouse bone marrow (BM) with the ability to form fibroblast colony-forming units (CFU-F) and differentiate into adipocytes, chondrocytes and osteocytes (2). Although MSCs have been isolated and characterized from BM (3), they could be also obtained from other sources such as amniotic membrane (4), skin (5), hair follicles, dental pulp (6, 7), adipose tissue (AT) (8, 9), umbilical cord blood (10), the endometrium, amniotic fluid (11), fetal liver, the placenta (PL) and the synovium (5). MSCs are defined by their plastic adherent growth, self-renewal and subsequent expansion under specific culture conditions and their differentiation potential *in vitro* and *in vivo* (12). Positive markers have been used such as CD44, CD90, CD73, CD105, and MHC-I; and negative markers such as CD14, CD34, MHC-II and CD45 as minimal criteria for characterization of MSCs have been established by the International Society for Cellular Therapy (ISCT) (13). Although adult BM and AT are the main sources for clinical use there are some drawbacks (14, 15). To harvest MSCs from AT there are limitation such as: the need to perform liposuction (16)

and enzymatic treatment (17). In the terms of ICBM, the MSCs population makes up only 0.01-0.0001% of the cellular content, and decreases when age increases (18). In addition, harvesting procedure is invasive. Therefore, alternative sources have been strongly pursued and recently, the reamer/irrigation/aspirator (RIA) device by continuous irrigation and aspiration of bone marrow during reaming of long bones has been providing a viable source to derive large numbers of MSCs called RIA-MSCs (19). RIA-MSCs are considered most suitable because it is waste during surgery and available in large quantities.

In addition to above-mentioned benefits, cell proliferation efficiency and differentiation potential of different sources should be consider as criteria to choose appropriate sources. Thus in this study we have isolated MSCs from ICBM, BM derived by the RIA device, and adipose tissue. We have examined cell expansion, surface markers, and multi-differentiation potential from

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three different donors for each source under the same condition.

Materials and Methods

Isolation of human MSCs from different sources

The Ethical Committee of the Medical University of Mashhad has approved this research. All patients included in this study were informed regarding the use of their samples for experimental analysis.

Iliac crest bone marrow (ICBM) MSCs

Human MSCs were isolated from 10 ml of iliac crest bone marrow taken from three healthy humans undergoing bone marrow harvest. Cell suspensions were diluted two times with phosphate buffered saline (PBS) and layered onto the Ficoll-Hypaque gradient (Cederlan, Cork, Ireland). After 20 min of centrifugation at 1800 g, the interface rings containing mononuclear cells (MNCs) were collected into individual tubes and washed twice by centrifugation in PBS. The resultant cell precipitate was resuspended in culture medium and transferred to T75 flasks. The medium contained DMEM with low glucose content, 100 U/ml penicillin, 100 µg streptomycin, and 10% fetal bovine serum (FBS). Medium was changed after 48 hours.

Adipose-derived tissue (AT) MSCs

Adipose-derived stem cells were obtained from three healthy human lipoaspirates (Razavi Hospital, Mashhad, Iran) based on our previously published protocol (20). Briefly, lipoaspirates were washed with sterile PBS to remove red blood cells and contaminating debris. Washed adipose tissue was treated with 1 mg collagenase (type I, Invitrogen, Carlsbad, USA), 10 mg of BSA (Biowest, Nuaille - France) and 2 mM CaCl₂ in 1 ml PBS per 3 ml lipoaspirate for 45 min at 37°C with gentle agitation. After centrifugation, the supernatant was discarded and the pellet containing adherent pluripotent cells, including MSCs, was cultured in DMEM low glucose containing 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, USA), and 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA). Medium was changed after 48 hours.

Reamer-irrigator-aspirator (RIA) MSCs

The RIA-MSCs were isolated from three patients with long bone fracture subjected to surgery. Technical guidance on MSC isolation of RIA has been previously reported (21). Briefly, mononuclear cells containing MSCs were separated from RIA by centrifugation over a Ficoll-Hypaque gradient (Cederlan, Cork, Ireland) and suspended in DMEM low glucose containing 10% FBS (Thermo Fisher Scientific, Massachusetts, USA), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, USA). Medium was changed after 48 hours.

Culturing of bone marrow, adipose tissue and reamer-irrigator-aspirator MSC

The medium with none adherent cells was discarded and carefully washed in PBS, and then culture medium was replaced with a fresh medium, which was replaced every 2-3 days. After attaining a subconfluent state, the

cells were removed with trypsin-EDTA (Thermo Fisher Scientific, Massachusetts, USA). Cultures of passage three were used for all experiments.

Functional assays for ICBM, AT and RIA-MSC enumeration

Colony-forming unit-fibroblast (CFU-F) assay was used for MSC enumeration (22). It was performed in triplicates in T75 flask at the cell seeding density of 3×10^5 cells/cm² from primary culture in DMEM low glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS (all from Thermo Fisher Scientific, Massachusetts, USA). Cultures were fed twice weekly, and colonies were scored on day 10 (23).

Multilineage differentiation assays

Osteogenic differentiation

For osteogenic differentiation assay, cells from three donors were seeded (10,000 cells/cm²) in 6-well plates and cultured in low glucose DMEM (Invitrogen, Carlsbad, USA) with 10% FBS, 100 nM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (all from Thermo Fisher Scientific, Massachusetts, USA) (24). Osteogenic differentiation was verified on days 7, 14 and 21 concerning calcifications produced in the extracellular matrix of differentiated cells by staining with Alizarin Red and confirming through the ALP activity of osteoblasts (25).

Chondrogenic differentiation

Chondrogenic differentiation was induced using the pellet culture technique (26). Briefly stated, approximately 1×10^6 cells/mL cells were placed in a 15-mL polypropylene tube (Falcon), and centrifuged to pellet. Then, 0.5 mL of chondrogenic basic medium (including high glucose DMEM with 1% ITS + Premix (BD Biosciences, BD Bioscience, New Jersey, United States), 40 µg/mL proline (Thermo Fisher Scientific, Massachusetts, USA), 100 nM dexamethasone, and 50 µg/mL ascorbic acid-2-phosphate) was added. It was freshly supplemented with 10 ng/mL of transforming growth factor-β₃ (TGF-β₃) (Peprotech, USA) to induce differentiation of RIA-MSCs within the pellet towards chondrocytes. Medium was changed every 3-4 days for four weeks. Pellets cultured in basic medium without TGF-β₃ were used as controls. After four weeks, the pellets were fixed in 10% (v/v) neutral buffered formalin, dehydrated in a series of ethanol, embedded in paraffin, and cut into 7µm sections. After deparaffinization, the sections were evaluated by Alcian Blue, Toluidine Blue, and Safranin O staining to detect glycosaminoglycans (GAGs) and proteoglycans resulting from chondrogenic differentiation (26-29).

Flow cytometric analysis (FACS)

The MSCs isolated from the three different sources were cultured on tissue culture-treated flasks. After reaching to 90% confluence, they were digested with 0.25% trypsin/ EDTA and washed with PBS containing 5% FBS. Suspensions were divided into aliquots in 1×10^6 MNCs per vial and incubated for 45 min with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies: CD34, CD44, CD45, CD90, CD105, CD11b, and CD73. Flow

Table 1. Primer sequences used for Real-Time PCR.

Serial no.	Gene symbol	Primer sequences (5'-3')	Product size (bp)
1	GAPDH	5'-CATGTTTCGTCATGGGTGTGAAC-3' 5'-CACAGTCTTCTGGGTGGCAG-3'	178
2	Osteocalcin (OCN)	5'-CCTCACACTCCTCGCCCTAT-3' 5'-TGCTTGACACAAAGGCTGC-3'	111
3	Alkaline Phosphatase (ALP)	5'-CATGCTGAGTGACACAGACAAGAAG-3' 5'-TGGTAGTTGTGTGAGCATAGTCCA-3'	68
4	Collagen II (COL II)	5'-GGCAATAGCAGGTTACGTACA-3' 5'-CGATAACAGTCTTGCCCCACTT-3'	79
5	Collagen X (COL X)	5'-CAAGGCACCATCTCCAGGAA-3' 5'-AAAGGGTATTGTGGCAGCATATT-3'	70

cytometry was performed on a FACS BD Accuri C6 flow cytometer and the cells were stained with respective isotype controls, FITC-conjugated or PE-conjugated non-specific IgG, to assess background fluorescence (all antibodies from Exbio, Slovakia).

Population doubling time (PDT)

The previously isolated and characterized three populations of the MSCs taken from different sources (ICBM, AT and RIA-MSCs) were seeded into the T25 cm² culturing flask at a density of 5000 cells/cm² in triplicates. Cells were harvested upon reaching >90% confluence with trypsin/EDTA, and stained with Trypan blue. Then they were count with a Hemacytometer for up to six passages. The mean PDT for each cell type was calculated after every passage by the following formula (30):

$PDT = T \times \lg 2 / (\lg N^t - \lg N^0)$, T=culture time (day), N⁰=initial cell number, N^t= harvested cell number.

RT-PCR

Total RNA was extracted from MSCs with Trizol reagent (Invitrogen, Carlsbad, USA), and cDNAs were synthesis by reverse transcription of 1 µg of cellular RNA (Thermo Scientific, Vilnius, Lithuania), according to the manufacturer's instruction. The PCR was performed for 40 cycles, with each cycle consisting of denaturation at 95°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s (the sequences of primers were used according to Table. 1). The PCR products were separated by electrophoresis on 1.5% agarose gel, stained with gel red (Biotium, Hayward, United States) and visualized under UV light.

Real time PCR

To quantify the differential gene expression program of human MSC during osteogenesis [alkaline phosphatase (ALP), Osteocalcin (OCN)] and chondrogenesis [Collagen II (COL II), Collagen X (COL X)] RT-PCR assay was performed with a Bio-Rad CFX96 thermal cycler and Syber Green Universal Master Mix (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. The thermal profile for Real time-PCR (94°C for 30 seconds, 60°C for 40 seconds, and 72°C for 30 seconds) was run for 40 cycles after an initial single cycle of 94°C for 15 min (all experiments were performed from three donors and each test was at minimum duplicated). The sequences of primers were used according to Table 1.

Statistical analysis

Results (mean ± SD) were reported as three inde-

pendent experiments. Statistical analyses were performed by the GraphPad Prism 6 using the one-way ANOVA, with Dunnett's multiple comparisons test.

Results

Characterization of MSCs from ICBM, AT and RIA

To determine whether RIA-MSCs share stem cell properties with ICBM-MSCs or AT-MSCs, we compared their morphology and surface markers. Flow cytometer (FACS) analysis showed that all MSCs were positive for the expression of the cluster of differentiation (CD) markers including CD44, CD73, CD90, and CD105, but nearly negative for CD11b, CD34 and CD45. We did not find any significant differences among the three sources for positive and negative markers (Figure 1). Besides, in passage 3, RIA-MSCs exhibited a spindle-shaped morphology comparable to that of ICBM- and AT-MSCs (Fig. 2).

Colonogenic ability and cellular senescence

Comparisons of the colonogenic ability of MSCs from different sources were evaluated by CFU-F assay. More colonies formed from RIA-MSCs (25.6 ± 20.8) after 10 days rather than from ICBM-MSCs (8.5±1). Colony formation was not obvious in AT-MSCs, in part due to debris of adipose tissue and oil droplets (Fig. 2A). However, AT-MSCs were expanded all over the flask surface area.

The cell proliferation rate or cellular senescence was also determined by evaluating PDT in all isolated MSCs up to passage 6 (Fig. 2B). The RIA-MSCs exhibited the greatest expansion capacity (2.6±0.5 days), whereas AT-MSCs (4.2±0.1 days) and ICBM-MSCs had the longest doubling time (4.9±0.4 days). The final PDT of the RIA-MSCs was found to be significantly higher than the ICBM- and AT-MSCs (Fig. 2B).

Osteogenic and chondrogenic lineages of MSCs

To investigate the differentiation capacity of the derived MSCs, cells were cultured in osteogenic and chondrogenic induction media. Differentiation potential in all MSCs was tested by staining for the typical lineage marker. Osteogenesis was defined by a bone-type marker, i.e., alkaline phosphatase (ALP) activity and deposition of calcium in extracellular matrix (ECM). Higher alkaline phosphatase activity and calcified extracellular matrix was detected in RIA-MSCs when compared to ICBM and AT-MSCs in response to osteogenic induction after days 7, 14 and 21 (Fig. 3A-L and Fig. 4 A-L). Chondrogenesis, characterized by an increase in proteoglycans and GAGs, was shown by Safranin O, Alcian

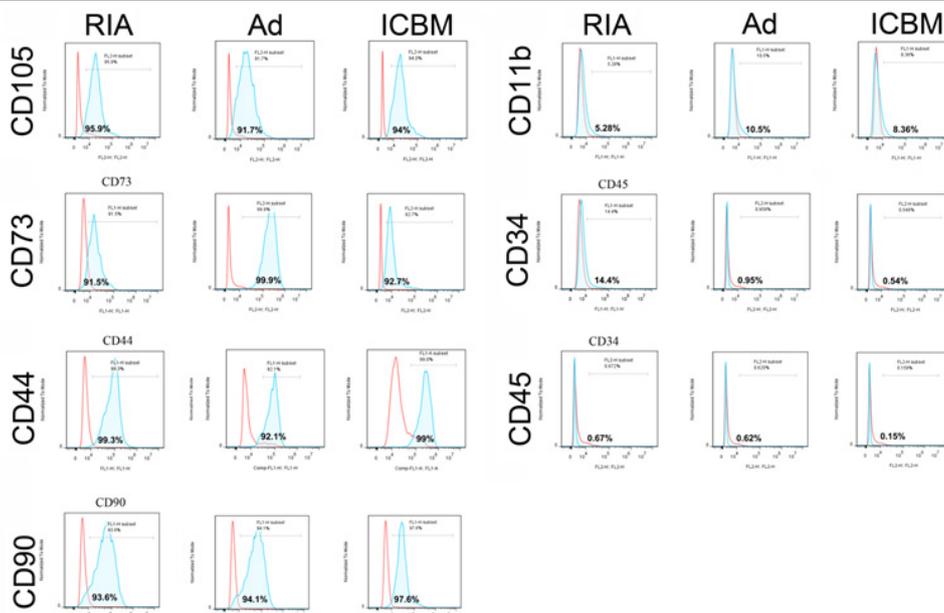


Figure 1. Expression pattern for cell surface markers of MSCs from ICBM, Ad and RIA cells. (A) Flow cytometry analysis was done for profile characteristic markers of MSCs. ICBM-, Ad-, RIA-MSCs have shown the same immunophenotype. (B) Flow cytometry results for negative surface markers of MSCs were negative.

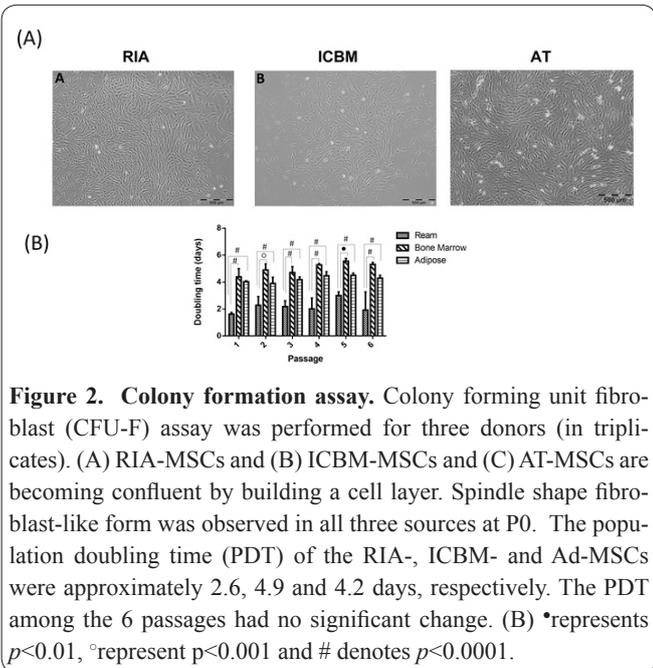


Figure 2. Colony formation assay. Colony forming unit fibroblast (CFU-F) assay was performed for three donors (in triplicates). (A) RIA-MSCs and (B) ICBM-MSCs and (C) AT-MSCs are becoming confluent by building a cell layer. Spindle shape fibroblast-like form was observed in all three sources at P0. The population doubling time (PDT) of the RIA-, ICBM- and Ad-MSCs were approximately 2.6, 4.9 and 4.2 days, respectively. The PDT among the 6 passages had no significant change. (B) *represents $p < 0.01$, °represent $p < 0.001$ and # denotes $p < 0.0001$.

Blue and Toluidine Blue staining. Qualitative analysis showed that chondrogenesis of RIA-MSCs are more intense (Fig. 5A-L) than AT- and ICBM-MSCs.

Furthermore, we analyzed the expression profile of MSCs taken from the three different sources for differentiation of specific markers of osteogenesis i.e., ALP (Fig. 3M-O) and osteocalcin (OCN) (Fig. 4M-O) and chondrogenesis (COL II, COL X) (Fig. 5M and N) by real time PCR. In terms of osteogenesis, there was significant difference of ALP between the RIA- and AT- than ICBM-MSCs ($p < 0.05$) at day 14 and 21 (Figure 3M-O). In contrast, there were no significant differences of OCN between the RIA-, ICBM- and BM-MSCs (Figure 4M-O). In terms of chondrogenesis, RIA-MSCs showed significant difference ($p < 0.05$) of Collagen II and Collagen X as compared with ICBM- and AT-MSCs (Figure 4M and N).

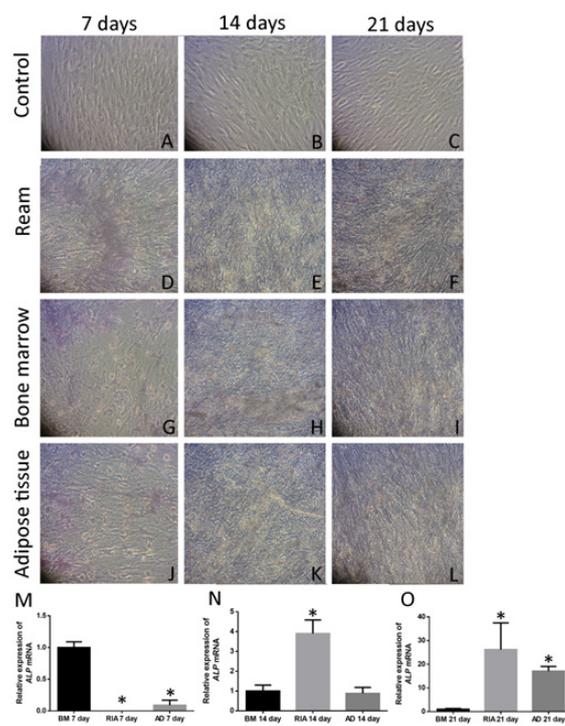
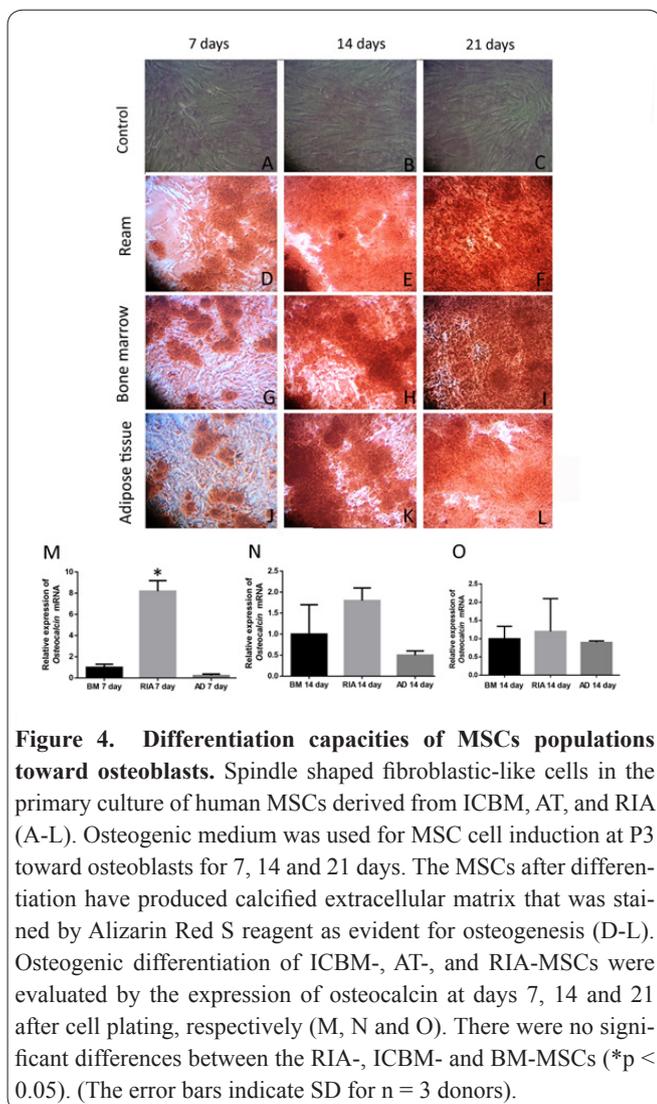


Figure 3. Differentiation capacities of MSCs populations toward osteoblasts. Spindle shaped fibroblastic-like cells in the primary culture of human MSCs derived from ICBM, AT and RIA (A-C). Osteogenic medium was used for MSC cells induction at P3 toward osteoblasts for 7, 14 and 21 days. Osteogenesis was confirmed by ALP assay of osteoblasts (D-L). Osteogenic differentiation of ICBM-, AT, and RIA-MSCs were evaluated by the expression of ALP at days 7, 14, and 21 after cell plating, respectively (M, N and O). There was significant difference between the RIA- and AT- than ICBM-MSCs (* $p < 0.05$). (The error bars indicate SD for $n = 3$ donors).

Discussion

The ICBM is the first source for MSC extraction and commonly used in clinical trials. However, this source



has some limitations including low frequency in number at bone marrow cells population in addition to low differentiation and proliferation with age. (31, 32). It is also a painful and invasive technique to obtain ICBM from patients for MSC use (4, 33). These disadvantages have encouraged researchers to find a better source to replace ICBM for isolating MSCs. Alternatively; AT-MSCs have advantages over ICBM, particularly considering increasing problems posed by obesity in recent years. In addition, AT-MSCs have a similar proliferative ability and differentiation potential to ICBM-MSCs (34).

Recently, another source of MSCs has been introduced termed RIA-MSCs. The RIA device was used to fix long bones with intramedullary nails. The ICBM that drains in this process is waste and several studies have showed that aspirate from the reaming system have large amount of MSCs (35, 36). The MSCs derived from RIA also have the ability to differentiate into multiple lineages, given the appropriate conditions (19, 21). In many studies the MSCs derived from different tissues have been shown to differentiate into cells in the mesodermal lineages such as osteoblasts, chondrocytes, and adipocytes (30, 37-39). In this study, we performed a comparison between three populations of MSCs derived from ICBM, AT, and RIA simultaneously. Human ICBM-, AT-, and RIA-MSCs, have shown global properties such as morphology and plastic adherence. Besides, expression of surface markers in MSCs and their dif-

ferentiation potential into osteogenic and chondrogenic lineages were evaluated to compare these sources for use in an allogenic therapy cell bank. So far, there have been no detailed studies comparing these MSCs derived from different sources. Based on the criteria defined by the International Society for Cellular Therapy (ISCT) (13), different markers have been used to characterized various-sourced MSCs. In this study, the CD90, CD34, CD44, CD105, CD45, CD73, and CD11b markers were tested. The MSCs from RIA, ICBM and AT under the same culture conditions have shown high positivity for CD90, CD44, CD73 and CD105, and negativity for CD34, CD45, and CD11b. However, our flow cytometric analysis revealed that RIA- and AT-MSCs have a more homogenous population than ICBM-MSCs.

We observed significant differences in the PDT, CFU-C, and proliferative potential among the three populations of MSCs; the RIA-MSCs exhibited the highest

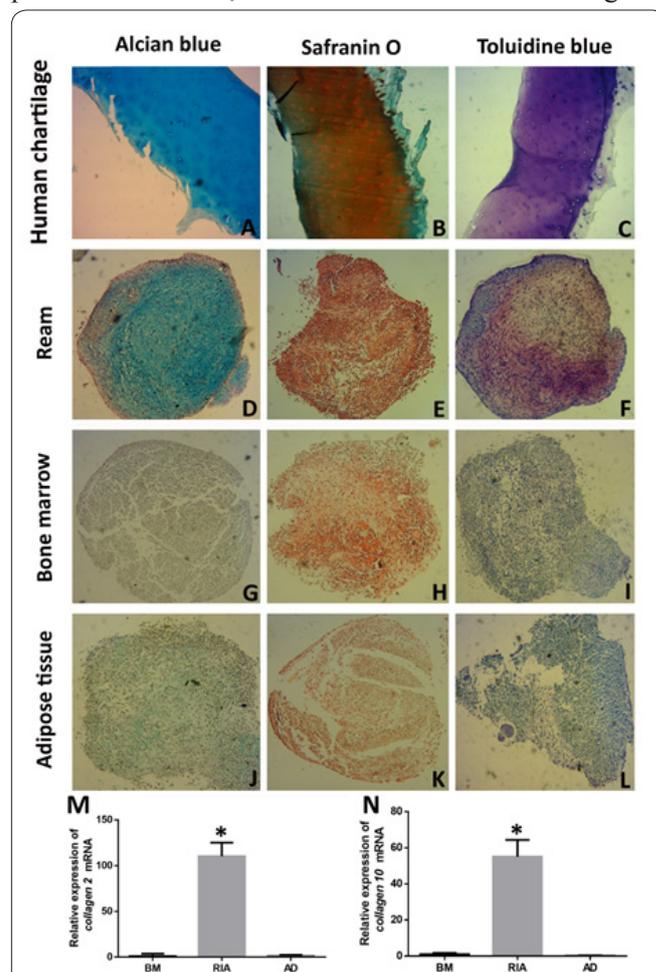


Figure 5. Differentiation capacities of MSCs populations toward chondrocytes. Cartilage differentiation of ICBM-, AT-, and RIA-MSCs were evaluated by the Alcian Blue, Safranin O and Toluidine Blue staining at 28 days. Human cartilage was the control; A: Alcian Blue, B: Safranin O and C: Toluidine Blue. Chondrogenic medium was used for MSCs induction at P3 toward chondrocytes for 28 days. MSCs after differentiation produced glycosaminoglycans (GAGs) and proteoglycans stained by Alcian Blue (D, G and J), Safranin O (E, H, and K) and Toluidine Blue (F, I, and L) are evident for chondrogenesis. Cartilage differentiation of ICBM-, AT-, and RIA-MSCs were evaluated by the expression of Collagen II and Collagen X at days 28 after cell plating, respectively (M and N). There was significant difference between the RIA- than ICBM- and AT-MSCs (* $p < 0.05$). (The error bars indicate SD for $n = 3$ donors).

growth rate. Presently, extensive literature exist about AT-MSCs and ICBM-MSCs (40, 41), but the growth diagram has shown that the proliferative capacity of the RIA-MSCs and AT-MSCs was significantly greater than ICBM-MSCs. These results are in agreement with another study that has compared ICBM and AT-MSCs (30). Recent studies have shown that yield of isolated MSCs from the waste of the RIA device were greater than ICBM (19, 21).

Our results have shown that there are significant differences between various populations of MSCs with respect to their differentiation potentials. The MSC differentiation into osteogenic and chondrogenic mesodermal lineage is an important feature that should always be considered to verify the plasticity of MSCs (40, 42). Importantly, RIA-MSCs undergo osteogenesis at a faster rate than ICBM- and AT-MSCs, based on the increase in ALP positive cells and calcium deposition in ECM (Fig. 3 and 4). Furthermore, cartilaginous pellets derived from RIA-MSCs displayed an ECM that stained positive for proteoglycans and GAG, higher than those seen in the cultures of ICBM and AT-MSCs. The ALP and OCN are important genes associated with the osteoblast phenotype and quantitation of gene expression using RT-PCR showed great synthesis of ALP about the RIA-MSC and AT-MSCs than ICBM, but OCN expression was not significantly different among three sources. This may be, in part, attributed to the fact that the initial cells isolated from RIA-MSCs have a higher frequency of osteoprogenitor cells than ICBM- and AT-MSCs. Our findings are consistent with other reports (21, 36, 43). In terms of chondrogenic differentiation, the expression of COL II and COL X were measured in three sources and there were high expressions in RIA-MSCs than ICBM and AT-MSCs. Thus, RIA-MSCs might be considered a better source for adult human cartilage repair. This finding may render RIA-MSCs as source of cells for orthopedic tissue engineering, which require suitable cells to produce abundant ECM synthesis. The RIA-MSCs can be used in patients suffering from non-union bone fracture or any other orthopedic problems. On the other hand, AT-MSCs might be best source in fatty patient, as the differentiation potential and cell growth were higher than ICBM.

Three different sources, i.e., human bone marrow derived MSCs from iliac crest and long bones by the RIA device, and adipose tissue, were compared for surface antigen markers, proliferative rate, and osteogenic and chondrogenic differentiation ability. Our findings showed that MSCs from various sources might have different therapeutic potential. The RIA-MSCs have high ability to expansion and high differentiation potential. Therefore, primitive RIA-MSCs have biological advantages in comparison to ICBM and AT-MSCs, making long bones a useful source for clinical application in bone and cartilage cell therapy as well as tissue engineering.

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