

Original Article

Use of Some Additives for Improving Mesenchymal Stem Cell Isolation Outcomes in Non-Mobilized Peripheral Blood

Noushin Pouryazdanpanah, MSc¹; Reza Vahidi, PhD^{2,3}; Shahriar Dabiri, MD⁴; Ali Derakhshani, PhD^{2,4}; Alireza Farsinejad, PhD^{1,3*}¹Department of Hematology and Laboratory Sciences, Faculty of Allied Medical Sciences, Kerman University of Medical Sciences, Kerman, Iran²Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran³Cell Therapy and Regenerative Medicine Comprehensive Center, Kerman University of Medical Sciences, Kerman, Iran⁴Pathology and Stem Cell Research Center, Kerman University of Medical Sciences, Kerman, Iran**Abstract**

Background: The mesenchymal stem cells (MSCs) of peripheral blood (PB) have been recognized as a promising source for allogeneic cell therapy. The objective of the present study was to isolate and characterize MSCs derived from non-mobilized PB, and evaluate their differentiation potential.

Methods: The buffy coat mononuclear fractions of the PB were concentrated using the Ficoll-Paque density gradient centrifugation and were grown on primary and secondary culture media, respectively. The isolated cells were characterized using a multidisciplinary approach which was based on morphology, immunophenotyping, gene expression, and multipotentiality. Flow cytometry and Reverse transcription polymerase chain reaction (RT-PCR) were used to identify the expression of different MSC markers. Finally, after culturing in osteogenic and adipogenic induction media, the isolated cells were stained by Alizarin red and Oil-Red O.

Results: In spite of absence of any bone marrow stimulating factor, the isolation approach in this study yielded a rather homogeneous and spindle-shaped mononuclear cell population (the yield of passage 0 was 0.65 ± 0.15) that stained positive for CD90, CD105, and CD73, and were negative for CD45 and CD34. These cells have high proliferative capacity (confirmed by the expression of Oct-4, Nucleostemin, and Nanog genes) and were able to differentiate into lineage-specific committed cells, when exposed to the appropriate medium.

Conclusions: Overall, it can be concluded that conventional, labour-intensive and time-consuming approaches are not necessary in isolating MSCs from PB. This relatively accessible and minimally invasive source, PB, represents a good alternative reservoir of homogeneous MSCs that could open a new era for practical exploitation in regenerative medicine.

Keywords: Differentiation, Mesenchymal stem cells, Peripheral blood, Regenerative medicine

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Introduction

Nowadays, the isolation of unspecialized stem cells with the capacity to differentiate into numerous cell types is attracting significant interest in regenerative medicine and tissue engineering.¹⁻⁴ In this context, the identification of new stem cell sources, minimally invasive isolation procedures and optimized cell culture conditions are needed for clinical applications. As a result of the effective clinical features of mesenchymal stem cells (MSCs), numerous studies have focused on isolation and differentiation of these multipotent cells.⁵⁻⁷ Extraction from the bone marrow (BM; as a main source) is an invasive and a high risk approach that yields a small and heterogeneous population.⁵ To circumvent these problems, researchers have attempted to separate a significant number of MSCs from alternative accessible tissue sources.^{8,9} Despite accessibility and high differentiation

potential of peripheral blood (PB) stem cells,⁸ growth factor-dependent strategies for the mobilization of bone marrow stem cells are time consuming, unaffordable and have side effects (nausea and vomiting).¹⁰ With these in mind, the feasibility of a new non-mobilized approach to separate an optimal quantity of blood-derived MSCs (which can be differentiated into specialized cells) was evaluated in this study.

Materials and Methods**Isolation and Expansion of Peripheral Blood Mononuclear Cells**

After obtaining informed consent, blood samples (10 mL) were obtained from 12 healthy young females. Since the pre-enrichment of mononuclear cells is highly recommended to improve the recovery of rare stem cells, density gradient centrifugation was used.

*Corresponding Authors: Ali Derakhshani, PhD; Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran. Tel/Fax: +98-34-33257318, Email: Derakhshani.sci@gmail.com; Alireza Farsinejad, PhD; Cell Therapy and Regenerative Medicine Comprehensive Center, Kerman University of Medical Sciences, Kerman, Iran . Tel/Fax: +98-34-33257318, Email: farsinezhad239@yahoo.com.

For this purpose, acid-citrate-dextrose (ACD)-treated blood was centrifuged (3500 rpm, 20 minutes), and the obtained buffy coat was diluted (1:1) with phosphate-buffered saline (PBS, pH 7.4; Gibco, BRL) and layered on the Ficoll-Paque solution (Biosera, France). After centrifugation, the isolated mononuclear cells were plated out in a growth medium containing Dulbecco's Modified Eagle's medium (DMEM)-F12 (Gibco, BRL), 15% Fetal bovine serum (FBS; Gibco, BRL) and streptomycin-penicillin antibiotic (Gibco, BRL) at a seeding density of 5×10^5 cells/cm². When the first colony of cells appeared (third day), the previous medium accompanied by non-adherent cells were evacuated and a new enriched medium containing fetal calf serum (5%; Gibco, BRL), L-glutamine (2.5 mM; Gibco, BRL), acidic fibroblast growth factor (5 ng/mL; R&D), Basic fibroblast growth factor (10 ng/mL; R&D), insulin (5 mM; Gibco, BRL) and heparin (15 U/mL; Gibco, BRL) were added to the cells for better and faster proliferation. It should be noted that the concentrations of acidic and basic fibroblast growth factors were selected based on the previous studies (1,2), and were confirmed by our evaluations. A phase contrast microscope was used for regular monitoring of cultured cells. The desired confluency (80%) was reached on the 5 – 8th days. The attached cells were obtained and further expanded on T25 flasks containing DMEM-F12, FBS (15%), and antibiotics (1% penicillin-streptomycin) for an additional 12–13 days. The culture medium was replaced twice a week and serial passages were performed using trypsin enzyme (Gibco, BRL).

Immunoprofiling

To assess the immunoprofile of isolated cells (passage 3), flow cytometry and anti-human antibodies were used. The cells were suspended in phosphate buffered saline (PBS) and then stained (30 minutes, 4°C) with the anti-CD45 (20 µL; 555482), -CD34 (20 µL; 555824), -CD73 (5 µL; 561254), -CD105 (5 µL; 561443) and -CD90 (5 µL; 555596) antibodies, all from BD-Biosciences, USA. An appropriate isotype-matched control antibody was used in all analyses. From each sample, 10000 incubated cells were analyzed with FACSCalibur flow cytometer (BD-Biosciences, USA) and Cellquest Pro software (version 5.1).

Molecular Investigation

Cells of the third passage were trypsinized, and then the RNA which was extracted from these cells (by Trizol reagent; Ambion, USA) was transcribed to cDNA by using a Thermo cDNA synthesis Kit (Thermo Fisher Scientific, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a PCR Thermal Cycler device (Thermo Fisher Scientific, USA). The PCR products were electrophoresed on 2% agarose gel, and then photographed under UV light. The used primers are presented in Table 1. Real-time PCR analysis was also carried out using Applied System device (Thermo Fisher Scientific, USA) and Ampliqon master mix (Ampliqon, Denmark) to compare the expression level of self-renewal genes (Nanog, Nucleostemin, and Oct-4) between MSCs isolated from two different tissues, PB and BM. Briefly, 5 µL cDNA and 5.5 µL water were added to 13.5 µL of the real-time PCR mix containing 12.5 µL master mix and 1 µL of each of specific primer pairs. PCR was initiated at 95°C for 15 minutes, and then continued with 40 cycles of 20 seconds at 95°C and 30 seconds at 72°C. To test the amplicon specificity, the melting curve was estimated. A non-template mixture was used as negative control. Finally, it should be noted that the protocol used for MSC extraction from BM was adapted from a previous study.¹¹

The differences of gene expression level between MSCs isolated from PB and BM were tested by Independent Samples t-test using SPSS 16.0 software package. The level of significance was considered as $P < 0.05$.

Assessing Differentiation Potential

The cells obtained were assessed for osteogenic and adipogenic differentiation potentials. Cells of passage 3 (150×10^3 /cm²) were seeded in 60 mm dishes and grown as a monolayer in DMEM-F12 and FBS until reaching the desired confluency (80%). At this point, the cells were incubated with the appropriate osteogenic (for 21 days) or adipogenic (for 14 days) differentiation medium, and culture medium exchanges were performed three times a week. Besides high glucose-DMEM (4.5 g/mL; Gibco, BRL), FBS (2%; Gibco, BRL), 50 µg/mL ascorbic acid 2-phosphate (Sigma, Germany) and 100 nM dexamethasone (Sigma, Germany) that are common compounds of various differentiation

Table 1. List of Primers for Indicated Genes

Molecular Markers	Forward Primers (5'-3')	Reverse Primers (5'-3')
Nanog	TGATTTGTGGGCTGAAGAA	AGTGGGTTGTTGCCTTTGG
Nucleostemin	GTGATTGAAGCCTCCGATGT	AGCCAGTCTCAAAGGCTC
Oct-4	TTACAGAACCACACTCGGAC	AGTGAGAGGCAACCTGGAGA
β-Actin	GGGCATGGGTGAGAAGGATT	CGCAGCTCATTGTAGAAGGT

media, osteogenic and adipogenic differentiation were induced using β -glycerol phosphate (10 mM; Sigma, Germany) and indomethacin (50 μ g/mL; Sigma, Germany), respectively. As a control, cells were grown in DMEM-F12 and FBS (2%). To confirm the adipogenic and osteogenic differentiation, cells were fixed (10 min, at room temperature) with PBS-buffered formalin (10%), and stained (1h) with Oil-Red O (Sigma, Germany) and Alizarin Red (Merck, USA) to detect intracellular lipid droplets and mineralized bone-like structures, respectively. Pictures from microscopy were taken with a Motic AE31 microscope (Hong Kong, China).

Results

Isolation and Morphological Analysis of MSCs

After the separation of mononuclear cells (approximately 10-15 million mononuclear cells from 10 mL of PB), they were grown in a growth medium containing DMEM, FBS, penicillin-streptomycin, and incubated in a CO₂ incubator (37°C). After the appearance of adherent cells, the new enriched medium was added to the cells. Cultures supplemented with a mixture of aFGF, bFGF, insulin and heparin increased the total number of spindle-shaped cells significantly. Then, the confluent adhesive cells were expanded for additional days. It was found that approximately $5-8 \times 10^6$ and $35-39 \times 10^6$ MSCs were obtainable at the end of passages 0 and 2, respectively. During the initial passages, morphological homogeneity was gradually achieved and a rather homogeneous population of MSCs was obtained after the second passage (Figure 1). Interestingly, isolation, expansion and growth of spindle-shaped MSCs were successfully carried out in all samples.

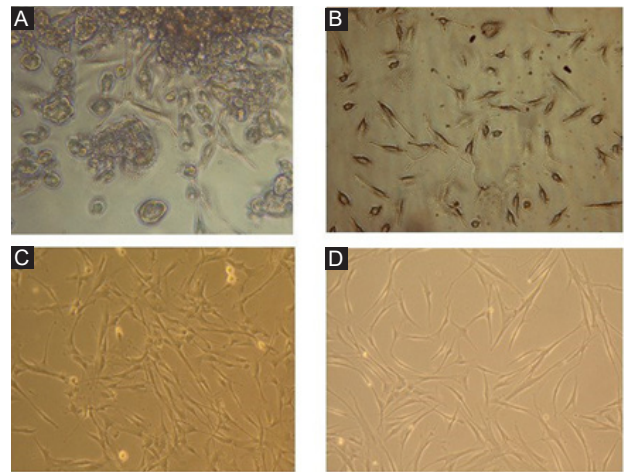


Figure 1. Morphology of Isolated Cells. With time, spindle-shaped fibroblast-like cells can be seen by phase-contrast microscopy. Interestingly, hematopoietic cell contamination gradually decreased during passages and was very low after passage 2. (A) Passage 0, (B) Passage 1, (C) Passage 2, (D) Passage 3. (Magnification: \times 200).

Immunoprofiling

The cells obtained were further characterized by their immunophenotype profile (Figure 2). The antigen profiling data revealed that these cells were negative for hematopoietic cell markers, i.e., CD45 (0.038 \pm 0.02%) and CD34 (0.293 \pm 0.22%), but were positive for MSC markers, CD90 (98.8 \pm 1.5%), CD73 (96 \pm 3.6%) and CD105 (95.47 \pm 2.3%).

Molecular Characterization of MSCs

Regarding pluripotency markers, isolated cells expressed Nucleostemin, Oct-4, and Nanog genes (Figure 3). Figure 4 shows the expression level of these markers in mesenchymal cells derived from PB and bone marrow in

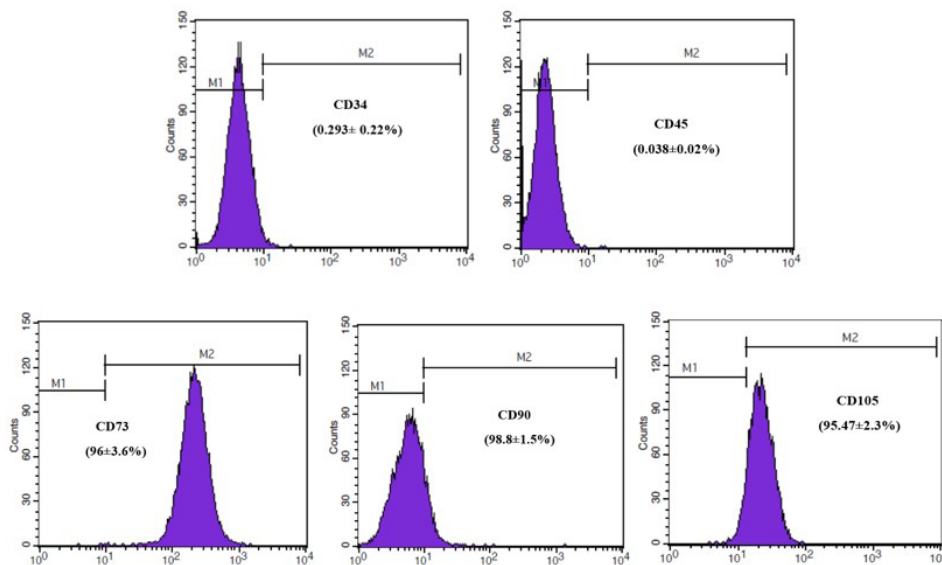


Figure 2. Immunoprofile of Isolated Cells. Representative flow cytometry histograms display the expression level of hematopoietic and mesenchymal stem cell markers by isolated cells (M2) compared with the isotype control threshold (M1).

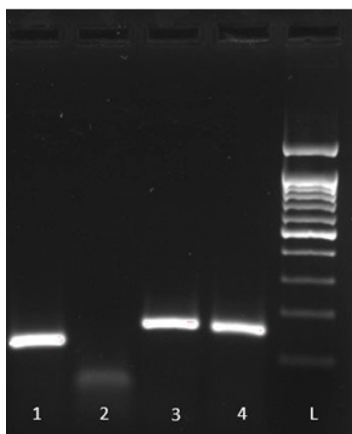


Figure 3. Representative amplification of the RT-PCR products. As shown, pluripotency markers, Nucleostemin (2; 80bp), Nanog (4; 155bp) and Oct-4 (3; 157bp), were expressed in RNA from the isolated cells. β -Actin (1; 117 bp) was used as an internal control gene. L: Ladder.

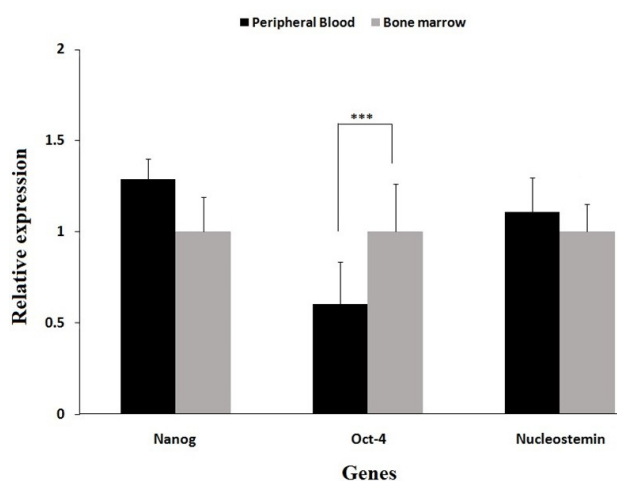


Figure 4. Comparative Expression Level of self-renewal markers by PB and BM-Derived Stem Cells. Results are presented as the mean expression level of markers. * $P < 0.05$, *** $P < 0.001$.

a comparative manner.

In Vitro Differentiation Into Adipocyte and Osteocyte

Isolated cells were further examined by their *in vitro* differentiation ability (a hallmark of MSCs) into osteocytes and adipocytes. For this purpose, early passage cells (passages 3-4) were cultured in the specific induction medium and evaluated by Alizarin Red and Oil Red O staining. The findings (Figure 5) indicate that isolated cells are able to differentiate into osteogenic (displays calcium deposits) or adipogenic (displays small lipid vacuoles) cells.

Discussion

MSCs are self-renewable, multipotent progenitor cells with the ability to differentiate into several mesenchymal

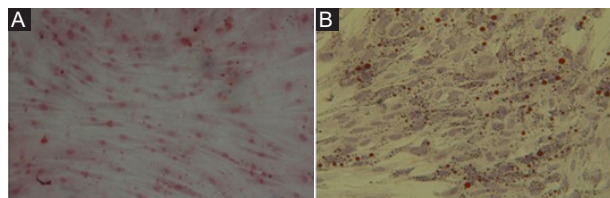


Figure 5. The Differentiation Potential of Isolated Cells. The extracellular calcium deposits; (A) stained orange-red color and (B) lipid vacuoles were evident in the Alizarin Red and Oil Red O stained histological slides. (Magnification: $\times 200$).

lineages.^{3,12,13} Despite great interest in the MSC therapy, the lack of accessible sources and well-defined extraction protocols are some limiting factors.¹⁴ The bone marrow is a convenient source of MSCs; however, the aspiration procedure is traumatic, painful and may have complications during extraction.^{15,16} Therefore, exploring alternative approaches for isolation of MSCs from non-invasive sources is growing in importance. PB is one of these sources that contains mobilized MSCs from the bone marrow. Major clinical trials have been conducted to identify replicable and efficient techniques for the isolation of MSCs from this source. However, the results obtained so far have been controversial.¹⁷⁻¹⁹ To solve the problem, studies have described the BM mobilization approach by using a growth factor treatment.²⁰ Non-specific mobilization and unknown consequences of BM stimulation are significant limitations of this process.^{21,22} These limitations prompted us to evaluate a modified approach for obtaining MSCs from non-mobilized PB. For this purpose, blood samples were obtained from 12 healthy subjects. Mononuclear cells of the buffy coat layer were isolated by density gradient centrifugation on Ficoll-Paque and were cultured in the modified medium that differs in some key elements from already used media. The optimized medium contained insulin, heparin and some growth factors. After depletion of suspended and unwanted cells (round cells), a homogeneous population of adherent spindle-shaped fibroblast-like cells were seen (Figure 1). These adherent cells had high proliferative capacity (mean doubling time was about 48 hours) and fulfilled immunological and molecular criteria for MSCs. Flow cytometric analysis indicated that most cells of the third passage had mesenchymal origin (positive expression for CD90, CD105, CD73 and negative expression for CD45 and CD34) (Figure 2). It was observed that isolated cells express Nanog, Oct-4 and Nucleostemin regulatory factors which mainly show pluripotency and self-renewability (Figures 3 and 4). To confirm the multilineage differentiation potential, isolated cells were plated into specific osteogenic (for 21 days) and adipogenic (for 14 days) induction media, and then calcium deposits and lipid vacuoles were

demonstrated by Alizarin Red and Oil Red O staining, respectively (Figure 5).

In conclusion, the findings of this study confirmed the presence of “circulating” MSCs and the possibility of isolating them from non-mobilized PB, rapidly using a simple modified method. In addition, it seems that the subsequent morphology and function of these stem cells is determined by culture conditions. Although inconsistent results exist in the literature,²³⁻²⁵ the results of this study revealed that insulin, heparin and growth factor combination can promote the proliferation and cell division of MSCs without significantly affecting their immunophenotype and differentiation potential. These positive effects can be attributed to cell cycle acceleration (by Akt-cyclin D1 pathway), extracellular biomolecules activity, activation of G protein coupled receptors and inhibition of cellular senescence.²⁶ Isolated cells enabled the generation of a homogeneous population of MSCs to differentiate into various tissues, and also made PB the candidate of choice for various cellular therapy applications.

Authors' Contribution

AF and AD supervised all aspects of the work and proposed the original concept and designed the experiment. NP and RV participated in the data acquisition. NP performed the real-time PCR experiments and interpretation of obtained results. Interpretation of immunoprofiling was performed by SD, AD and NP contributed to the data analysis. All authors contributed to writing the manuscript. RV and SD provided critical reviews and revisions, and performed the referees' recommendations in order to promote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

Ethical approval was obtained by ethics committee of Kerman University of Medical Sciences, and experimental procedures were followed according to the presented guidelines. In addition, all blood samples were obtained following informed consent of the patients.

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