



Comparison of the efficacy of bone morphogenetic protein-4 on *in vitro* differentiation of murine adipose and bone marrow mesenchymal stem cells into primordial germ cells

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Abstract

Background and purpose: *In vitro* development of functional gametes from pluripotent stem cells is a promising prospect to treat infertility. Mesenchymal stem cells with a high degree of plasticity and less tumorigenicity are a reliable source of stem cells for the generation of gametes. The present study aimed to compare the differentiation potential in the mesenchymal stem cells that are derived from bone marrow (BMD-MSCs) and adipose tissue-derived mesenchymal stem cells (AD-MSCs) into germ cells in a culture medium containing bone morphogenetic protein-4 (BMP-4).

Experimental approach: In this study, MSCs were isolated from both bone marrow and adipose tissue of murine samples. To further verify the nature of the harvested stem cells, their multipotency and surface marker were examined. The identified stem cells were cultured in a medium supplemented with 0 and 25 ng/mL of BMP-4 for 4 days. Flow cytometry analysis, immunofluorescence staining, and real RT-PCR were used to assess the expression levels in germ cell-specific biomarkers (Mvh, Dazl, Stra8, and Scp3).

Findings/Results: CD44⁺, CD45⁻, CD31⁻, BMD-MSCs, and AD-MSCs showed to be capable of differentiating to osteo-adipogenic lineages. The flow cytometry, immunofluorescence, and RT-PCR results indicated that early germ cell markers (Mvh and Dazl) were expressed in both types of cells but they were significantly higher in BMD-MSCs than AD-MSCs.

Conclusion and implications: Based on our results, the addition of exogenous BMP4 to the culture medium could differentiate BMD-MSCs and AD-MSCs into primordial germ cells, but it is inadequate to further develop into late germ cells *in vitro*. Moreover, the results revealed that, although AD-MSCs were easier to collect and had faster growth and proliferation rates than BMD-MSCs, the BMD-MSCs were better capable of differentiation into primordial germ cells. They may serve to be considered a more suitable source of MSC for *in vitro* generation of gametes than AD-MSCs.

Keywords: BMP-4; Germ cells; Infertility; Mesenchymal stem cells.

INTRODUCTION

Infertility is a problem afflicting 10 to 15% of couples at the reproduction age (1).

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There have been many strategies to treat human infertility. However, Assisted Reproductive Techniques (ARTs) are not adequately suited for the treatment of all kinds of infertilities. *In vitro* generation of gamete from stem cells is a novel promising approach to treat infertility (2). Studies have shown that germ cells can be generated from embryonic stem cells *in vitro* (3). Regardless of the prominent differentiation capability of embryonic stem cells in comparison with adult stem cells, moral concerns and legislative limitations are the fundamental limitations of the embryonic stem cells hindering their clinical applications (4). In the field of regenerative medicine, adult stem cells with multilineage differentiation and self-renewal qualification showed to be an incredible guarantee for the treatment of various disorders (5,6). Mesenchymal stem cells (MSC) are adult stem cells offering new chances to treat certain diseases including infertility. Bone marrow- and adipose tissue-derived MSCs (BM-MSCs and AD-MSCs) are the most common sources of MSCs. Several studies proved the therapeutic potentials of these two sources of stem cells in multiple disorders (6). Various stimulating factors including bone morphogenetic protein-4 (BMP-4) have been used for differentiating MSCs into germ cells *in vitro* (7-9).

TBMP-4, from the transforming growth factor β family, has been found as a key regulator of embryonic primordial germ cell (PGC) differentiation (10). Its functional role in PGC differentiation during embryonic gonad development has been shown in the knockout mouse model (11).

Because AT is more accessible and has a higher differentiation potential than BM (12,13), in the current study, we compared the capability of differentiating in BMD-MSCs and AD-MSCs to germ cells by using BMP-4-supplemented culture medium.

MATERIALS AND METHODS

Isolating and culturing MSCs

All animal procedures related to the isolation of MSCs agreed with the ethical considerations of research with lab animals in Tehran

University of Medical Sciences (Ethical No. 90-02-30-12942). To obtain BM-MSCs, the femurs and tibias bones in 4-6-week male NMRI mice were aspirated and cultured based on the protocol provided by Jamous *et al.* under sterile conditions.

AD-MSCs were harvested from the fat around the testis of 6- to 8-week NMRI mice based on the above-mentioned protocol. In brief, the fatty tissue was cut into tiny pieces which were then incubated in a solution of collagenase (2%) inside an incubator at 37 °C for 1 h. Then, Dulbecco's modified eagle's medium (DMEM; Invitrogen, USA) containing 10% of fetal bovine serum (FBS; Invitrogen, USA), was incorporated to neutralize collagenase. The two cell lineages were centrifuged at 1500 rpm for 5 min in order to remove all cell debris. After discarding the supernatant, the pellet was resuspended in DMEM medium supplemented with FBS (10%), 10 IU/mL of penicillin, and 10 μ g/mL of streptomycin (Invitrogen, USA) and was incubated at 37 °C with 95% of humidity and CO₂ (5%).

To eliminate the cells that were not adherent, the culture medium was altered every 3 days and trypsin (Invitrogen, USA) was used for passage when the cells reached about 90-95% confluence.

Osteogenic and adipogenic differentiation

After the third passage, the osteogenic and adipogenic induction medium was used to culture BMD-MSCs and AD-MSCs (Bonyakhte Inc. in Iran) and were incubated at 37 °C with 5% CO₂, and 95% humidity for 21 days when the culture medium was replaced after 3-4 days. To evaluate the differentiation potential into osteoblast and lipoblast, Alizarin Red solution (Sigma, Germany) and Oil-Red-O (Merck, Germany) were used to stain the cells. In brief, PBS was used to wash the cells twice and 4% of paraformaldehyde was used to fix them for 20 min. After three times of washing in PBS, 500 μ L of Alizarin Red S solution and Oil Red-O were inserted into the cells. In 10 min, we washed the cells with distilled water and visualized them under a light microscope. The red dots showed differentiation into osteoblasts and adipoblast.

Immunophenotyping analysis

To affirm the BMD-MSCs and AD-MSCs existence, after the fourth passage, about 10^5 - 10^6 of both BMD-MSCs and AD-MSCs were harvested and the cell surface marker CD44 (ab25064, Abcam) was representative of MSCs, while CD45 (ab25670, Abcam) and CD31 (ab95652, 78T Abcam) were considered hematopoietic and endothelial stem cell indicators, respectively. They were analyzed using flow cytometry (Becton Dickinson FACS Calibur, Heidelberg, Germany).

For isotype controls, we used the fluorescein isothiocyanate (FITC) mouse IgG2a isotype control (11-4724, eBioscience, UK) and rat IgG1 isotype control (ab18412, Abcam) with a dilution ratio of 1:200.

MSCs differentiation into germ cells

In the experimental groups, 10^5 BMD-MSCs and AD-MSCs were separately cultured in the DMEM F12 containing 10% FBS, penicillin 10 IU/mL, streptomycin 100 μ g/mL, and 25 ng/mL BMP-4, and incubated for four days at 37 °C with 5% CO₂ and 95% humidity (8). In the control group, both AD-MSCs and BMD-MSCs were cultured in a conditioned medium without differentiation-inducing factors for 4 days. The medium was changed every 2 days. After this time, the cells were evaluated for germ cells identifying characteristics *via* immunofluorescence, flow cytometry, and real-time polymerase chain reaction (RT-PCR) techniques.

Flow cytometry analysis

In the present study, we used the flow cytometry analysis to assess germ cells characteristics markers, mouse vasa homolog gene (Mvh), and deleted in azoospermia-like (Dazl) according to the manufacturer's instructions (Chemicon). Briefly, after fixing the cells with 4% of paraformaldehyde, the cells were incubated in 5% of bovine serum albumin (BSA) for 8 min at room temperature. The primary anti-Dazl (Santa Cruz, sc-27333, USA) and anti-Mvh (antibodies-online, 701365) antibodies with a dilution proportion of 1:50 were incorporated into the cells, following three washes with PBS. The next day, the cells were placed into a secondary antibody solution that

consisted of FITC diluted in an ideal form including rat anti-rabbit IgG (antibodies-online, 30087, 1:100, Germany), as well as donkey anti-goat IgG (Santa Cruse, sc-2024, 1:100, Germany) after washing with PBS. To fix the confluent cells, paraformaldehyde (4%) was used at room temperature. FITC mouse IgG1 (eBioscience, 11-4714, UK) and rabbit polyclonal IgG (Abcam, ab27472, USA) with a dilution ratio of 1:200 were used as isotype controls. Finally, flow cytometry analysis (Becton Dickinson FACS Calibur flow cytometer, Heidelberg, Germany) was done, and the data were analyzed, using WinMDI 2.9 (J. Trotter 1993-1998, USA).

Immunofluorescence

To confirm the differentiation potential of the isolated BMD-MSCs and AD-MSCs toward germ-like cells, immunofluorescence staining was done using two germ cell-specific markers. In brief, cold PBS was used to wash 10^5 - 10^6 cells and 4% of paraformaldehyde was used to fix them for 10 min at room temperature. Then, we kept the cells for 15 min in Triton™ 100-X to make them permeable. The non-specific antibody binding was blocked using 1% of BSA in PBS/tween for 45 min. Then, the cells were incubated with a mixture of two different primary antibodies against Dazl and Mvh at a dilution of ratio 1:50 overnight at 4 °C. The next day, the cells were washed three times with PBS and incubated in a secondary antibody solution including rat anti-rabbit IgG (antibodies-online, 30087, 1:100) as well as donkey anti-goat IgG (Santa Cruz, sc-2024, 1:100, Germany) for 2 h at room temperature. The cells were washed twice with PBS to remove additional antibodies. To assess the expression of the germ cell markers, we incubated the cells in 5 mg/mL FITC conjugated. For isotype controls, the FITC mouse IgG1 isotype control (11-4714, eBioscience, UK, 1:200) as well as rabbit polyclonal IgG (ab27472, Abcam, USA, 1:200) were used. We also used 4',6-diamidino-2-phenylindole (DAPI) as the nuclear staining. The whole procedure was carried out in a dark room. The positive population for these markers appeared in a brilliant green color under a fluorescent microscope.

Table 1. Primer sequences.

Genes	Primer sequences	Amplicon length	GenBank code
<i>Mvh</i>	F:-5' CGAAACATAGGTGATGAAAGAAC R :-5' CCACTGAAGTAGCAACAAGAAC	193 bp	NM_001145885
<i>Dazl</i>	F:-5' ATCAGCAACCACAAGTCAAG R :-5' CAAATCCATAGCCCTTCG	188 bp	NM_001145885
<i>Scp3</i>	F:-5' AAAGCATTCTGGGAAATCTG R :-5' GTACTTCACCTCCAACATCTTC	188 bp	NM_011517
<i>Stra8</i>	F:-5' GGC AGT TTA CTC CCA GTC TG R: -5' TTC CTT GAC CTC CTC TAA GC	166 bp	NM_009292
<i>GAPDH</i>	F: 5'-AACTTTGGCATTGTGGAAGG-3' R: 5'-GGATGCAGGGATGATGTTCT-3'	132 bp	NM_008084

RT-PCR

Trizol reagent kit (Ready Mini Kit, Qiagen, USA) was used to extract the total RNA, as instructed by the manufacturer, and to clean up any DNAs in the residual culture flask, DNAase I (Fermentas, Canada) was used. cDNA synthesis was performed using a reverse transcription kit (Transcript first-strand cDNA synthesis; Roche, USA), based on the manufacturer's protocol. The reverse-transcribed products of *Dazl*, *Mvh*, stimulated by retinoic acid 8 (*Stra8*), and synaptonemal complex protein 3 (*Scp3*) were amplified by RT-PCR with SYBR Green (Takara, Japan) on an ABI RT-PCR system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Normalization of gene expression was done against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, as an internal control). Relative fold changes in mRNA expression were calculated via the $2^{-\Delta\Delta CT}$ method. The primer sequences that were utilized for RT-PCR have been shown in Table 1.

Statistical analysis

Data are reported as mean \pm standard deviation (SD). An analysis of variance (ANOVA) was used to evaluate the statistical significance, using SPSS 16.0 (SPSS Inc., Chicago, IL). The *P*-value < 0.05 represented significant differences.

RESULTS**Isolating and characterizing BMD-MSCs and AD-MSCs**

Spindle-shaped BMD-MSCs and AD-MSCs

(Fig. 1 A and B) were evaluated for mesenchymal differentiation. Osteoblast differentiation was assessed using Alizarin Red S staining. After 3 weeks of culture in the osteogenic medium, the red spots were observed in differentiated cells toward osteoblasts (Fig. 1 C and D). Also, Oil-red O was used to stain the cells and assess their differentiation into adipocytes. The accumulation of lipid droplets in cells was clear after a 3-week culturing in adipogenic medium (Fig. 1 E and F).

The flow cytometry findings confirmed the entity of MSCs. The flow cytometry data demonstrated that the cultured BMD-MSCs and AD-MSCs expressed CD44 markers (superficial characteristics of stem cells). In contrast, there was no evidence for CD31 (the endothelial marker) and the expression of CD45 (hematopoietic marker) (Fig. 2).

Differentiation of BMD-MSCs and AD-MSCs into germ cells

The results were presented in the experimental and control groups, to evaluate the differentiation induction.

RT-PCR was used to assess the expression of germ cell genes *Dazl*, *Mvh*, *Stra8*, and *Scp3*. According to the data collected through the quantitative RT-PCR, no expression was found for *Dazl*, *Mvh*, *Stra8*, and *Scp3* in the control group. Besides, the estimated values for the expression of *Dazl* and *Mvh* in BMD-MSCs were significantly higher than AD-MSCs, while no expression was found for *Stra8* and *Scp3* in both experimental groups (Fig. 3).

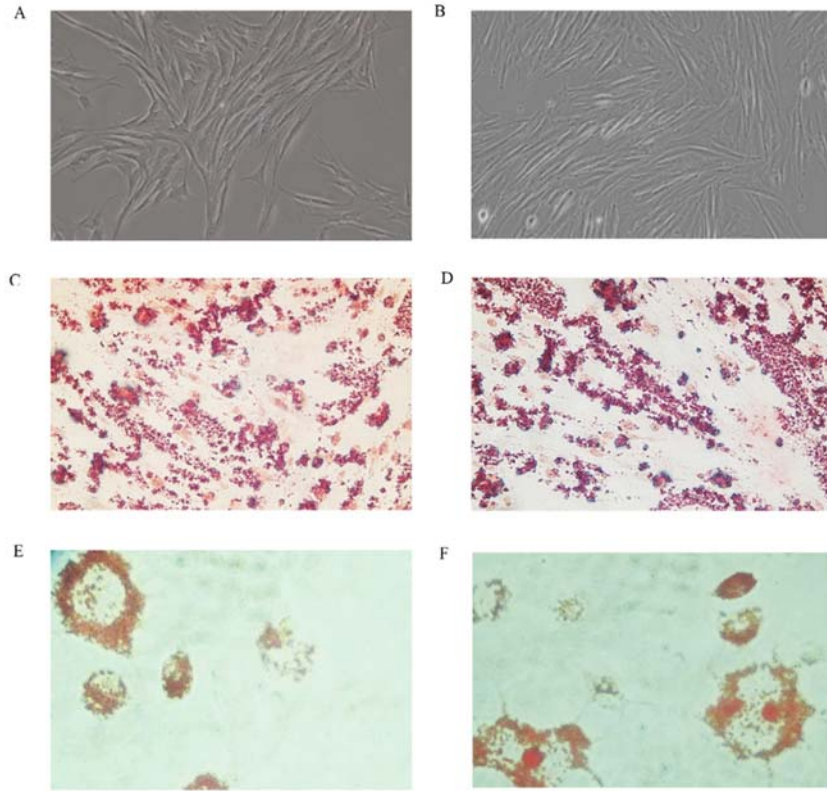


Fig. 1. (A) BMD-MSCs and (B) AD-MSCs morphology before differentiation after 3 passages (fibroblastic like cells); differentiation of (C) BMD-MSCs and (D) AD-MSCs to osteoblasts after Alizarin red staining; differentiation of (E) BMD-MSCs and (F) AD-MSCs to adipocytes after Oil Red-O staining. Original magnification, 400 \times . BMD-MSC, Mesenchymal stem cell-derived from bone marrow; AD-MSC, an adipose tissue-derived mesenchymal stem cell.

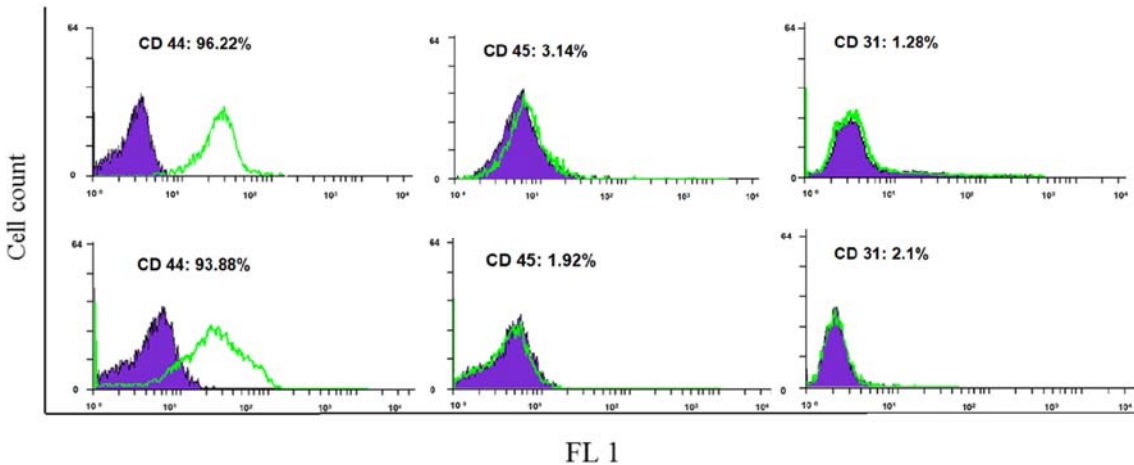


Fig. 2. Flow cytometry analysis for characterization of mesenchymal stem cells derived from bone marrow (upper) and adipose tissue-derived mesenchymal stem cells (lower). Expression of mesenchymal stem cells positive (CD44) and negative (CD45 and CD31) markers are illustrated. Isotype control is violet

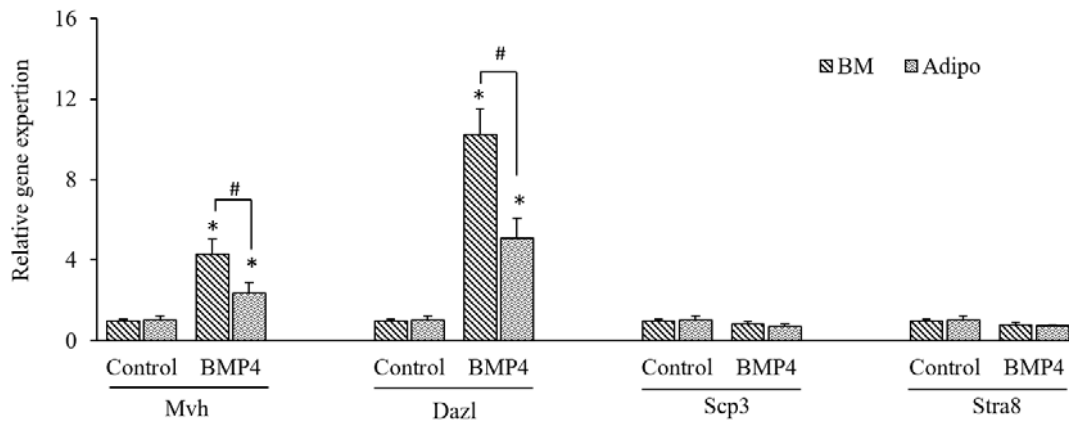


Fig. 3. Relative mRNA quantities of Mvh, Dazl, Stra8, and Scp3 in primordial germ cells derived from BMD-MSCs and AD-MSCs in BMP-4 along with the control group. * $P < 0.05$ Indicates significant differences compared to the corresponding control group and # $P < 0.05$ between the specified groups. AD-MSCs, Adipose tissue-derived mesenchymal stem cells; BMD-MSCs, mesenchymal stem cells derived from bone marrow; BMP-4, bone morphogenic protein-4.

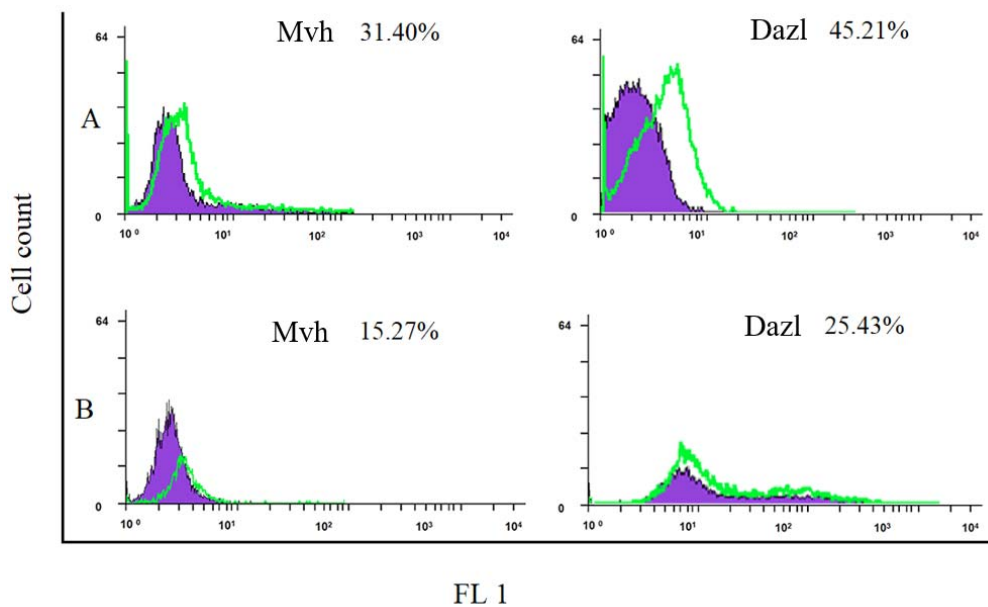


Fig. 4. Flow cytometry analysis of mesenchymal stem cells derived from bone marrow (upper) and adipose tissue-derived mesenchymal stem cells (lower) after culturing in the medium contains bone morphogenic protein-4 for 4 days. The date indicated that these cells are positive for the germ cell markers (Mvh and Dazl). Isotype control is violet.

In addition, flow cytometry and immunofluorescence revealed the expression of Dazl and Mvh in the experimental groups, but based on the flow cytometry results, the quantity of expression in the BMD-MSCs group was significantly higher than the AD-

MSCs (Figs. 4 and 5). Besides, no expression of germ cell characteristics (Dazl and Mvh) was detected through flow cytometry and immunofluorescence in the control group (Figs. 6 and 7).

c

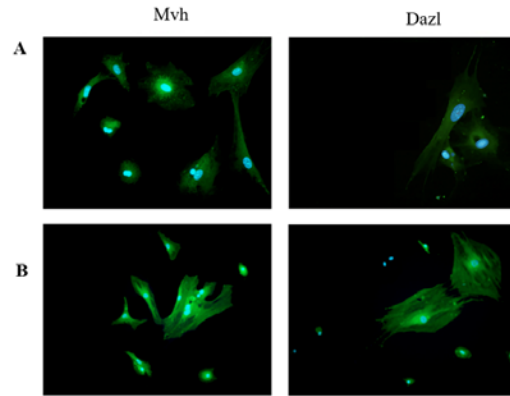


Fig. 5. Immunofluorescence staining of Mvh and Dazl (green) in the germ cells derived from (A) mesenchymal stem cells derived from bone marrow and (B) adipose tissue-derived mesenchymal stem cells after culturing in the medium contains bone morphogenic protein-4 for 4 days. The cores were stained by 4',6-diamidino-2-phenylindole (DAPI, blue). Both cell types were positive for the Mvh and Dazl antibodies.

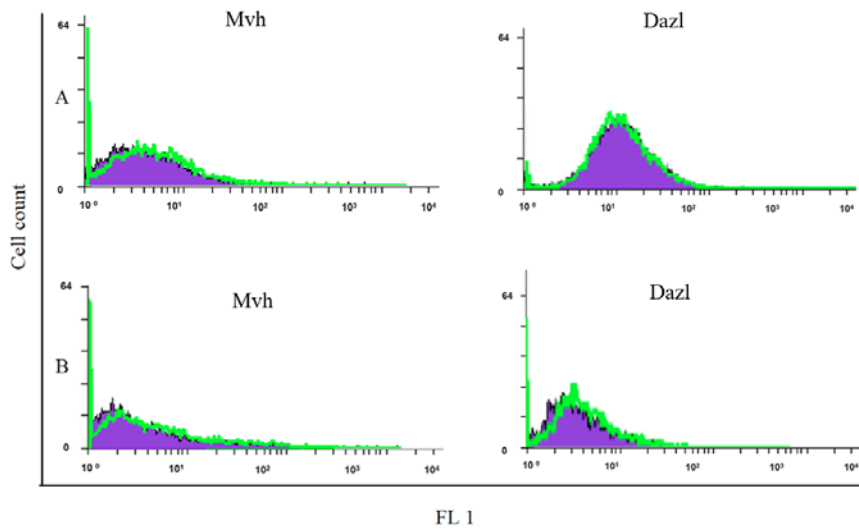


Fig. 6. Flow cytometry analysis of mesenchymal stem cells derived from bone marrow (upper) and adipose tissue-derived mesenchymal stem cells (lower) after culturing in a non-differentiation medium for 4 days after the fourth passage. These cells were negative to the germ cell markers (Mvh and Dazl).

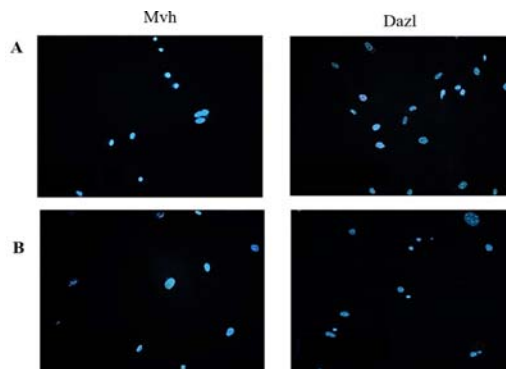


Fig. 7. Immunofluorescence staining of Mvh and Dazl in (A) mesenchymal stem cells derived from bone marrow and (B) adipose tissue-derived mesenchymal stem cells after culturing in a non-differentiation medium for 4 days. The cores were stained by 4',6-diamidino-2-phenylindole (DAPI, blue). Both cell types were negative for the Mvh and Dazl antibodies.

DISCUSSION

Innovative medical methods are used today for infertility. One common method is the use of stem cells. The existing research and developments in recent years in stem cell differentiation into different cells have changed scientists' attitudes to the fertility and infertility issue. Many developments have been made in treating infertility and also the *in vitro* production of gamete and cellular technology (3,14,15).

Different studies proved the potential differentiation of mesenchymal stem cells. The multi-potency of BMD-MSC and AD-MSC has made them a proper alternative to treat and control mesoderm defects and also the lineages of other tissues (16,17). Also, previous studies showed that BMD-MSC and AD-MSC could be differentiated under appropriate conditions to germ cells (15,18-22).

In accordance with the previous reports, the MSCs isolated in our study differentiated into adipoblast and osteoblast in differentiation medium were associated with cell multipotency (23). In general, the absence of blood and endothelial cell markers, and the presence of mesenchymal stem cell markers with high proliferation and differentiation potential indicate that the cells obtained in this study were a pure population of mesenchymal stem cells (20,24).

Having said that, the isolation of human BMD-MSCs is an invasive and painful procedure that requires the aspiration of bone marrow during general anesthesia. While AD-MSCs can be isolated more easily. It requires less painful procedures to isolate a relatively larger number of AD-MSCs with a high proliferative capacity (25,26). Similarly, our results demonstrated a rapid growth and proliferation rate in AD-MSCs compared to the BMD-MSCs.

The goal of this study was to find the best source of MSC differentiation potential into germ cells under BMP-4 induction as well as to compare their differentiation capability.

We showed that BMD-MSCs and AD-MSCs were able to express the markers specific to early germ cells (Mvh and Dazl) but not late

germ cells markers (SP3 and Stra8) *in vitro* under BMP4 induction.

However, several studies reported a spontaneous differentiation of stem cells into germ cells (27). The majority of other research findings were consistent with ours and indicated that MSCs cannot be differentiated into germ cells without any differentiating factor (28,29).

BMPs are key to regulating stem cell fate among mammals and PGC formation in the epiblast (29,30). Bmp-4 null mutants led to a drastic defect in germ cell development and a lack of PGCs (31).

Shirazi *et al.* suggested that BMP4 can induce differentiation of mouse pluripotent SSEA-1 cells isolated from BM into primordial germ cells *in vitro* (29). In addition, Mazaheri *et al.* demonstrated that adding exogenous BMP4 to culture medium could differentiate BMD-MSCs into PGCs (8).

We found that the germ cells that were derived from BMD-MSCs and AD-MSCs were able to express early germ-specific markers (Mvh and Dazl) but not SP3 and Stra8, *in vitro*.

Dazl, Scp3, and Stra8 are germ cell markers that are expressed in prenatal and postnatal stages in both spermatogenesis and the oogenesis process (32,33). Stra8 and Scp3 as meiosis markers are expressed in the ovary or testis environment in response to induction with retinoic acid during the development (34).

Among all germ cell markers, Mvh is one of the most reliable markers to detect germ cells lineage and move from post-migration stages to post-meiosis stages. This gene is a key to the proliferation and differentiation of primary germ cells (35).

Our results demonstrated that BMP-4 is not exclusive enough to induce BMD-MSCs and AD-MSCs to develop *in vitro* further into late germ cells. These findings were consistent with earlier research reports (8,29).

Based on our previous research, the combined addition of retinoic acid and BMP4 has led to the development of late germ cells *in vitro* (18). Consequently, it seems that to continue the development, more time or the use of other stimuli combined with BMP4 is needed.

Nevertheless, our results demonstrated that the expression of primordial germ cell's specific markers in the BMD-MSCs treated group were significantly higher, compared to AD-MSCs, and demonstrated a higher differentiation potential of BMD-MSCs. Previous studies indicated that BMD-MSCs have a better cell differentiation potential than AD-MSCs and there are valuable and reliable sources of MSCs compared with peripheral blood (36). However, BM stem cell harvesting is a highly invasive procedure with a very low stem cell number that leads to osteoporosis due to aspiration (37).

CONCLUSION

This study demonstrated that AD-MSCs and BMD-MSCs were able to be differentiated into primordial germ cells by adding exogenous BMP4 to the culture medium. Thus, to promote germ cell differentiation, long-term *in vitro* culture and also other meiosis inducers might be used for differentiation of AD-MSCs and BMD-MSCs. Our findings revealed that although AD-MSCs had a faster growth and proliferation rate than BMD-MSCs, the cells harvested from bone marrow are more potent to be differentiated into germ cells under *in vitro* conditions induced by BMP-4. It is clear that *in vitro*-generated germ cells proved to be an appropriate model and potential strategy to treat infertility.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

M. Hosseinzadeh Shirzeyli designed and performed the experiments and supervised the project; F. Aliakbary contributed to the writing of the manuscript in consultation with other authors and verified the analytical methods; F. Ghasemi contributed to the implementation of

the research and verified the analytical methods; F. Eini analyzed the data; F. Hosseinzadeh Shirzeyli conceived the presented idea, contributed to the experiment, and provision of study materials, reagents, materials, laboratory samples, animals; F. Vanaki contributed to the writing of the manuscript and revision; A. Sobhani was involved in planning and supervised the work. The final version of the manuscript was approved by all authors.

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