

Original Article



Evaluation of isolation method in remaining of differentiation potential of perivascular human umbilical cord mesenchymal stem cells toward male germ cell-like

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Abstract

Background and aims: Infertility is one of the most common problems among couples. Generation of male germ cells from adult stem cells is a current promising priority of researchers. This study aimed to investigate the potential of human umbilical cord mesenchymal stem cells (hUMSCs) on the expression of male germ cell markers after isolating by this method.

Methods: The hUMSCs was incubated with retinoic acid, testosterone, and conditioned medium (prepared from testicular cell cultures of 7-day-old mice) during 3 days. The bands were visualized and densitometry was accomplished using LI-COR Biosciences software.

Results: The high expression levels of C-KIT, DAZL, PIWIL2, and DDX4 in mRNA and protein levels were observed in treated hUMSCs.

Conclusion: Results of reverse transcription polymerase chain reaction (RT-PCR) and western blotting showed that method of isolation had no adverse effects on differentiation potential of hUMSCs.

Keywords: Infertility, Human umbilical cord mesenchymal stem cell, Male germ cell, Retinoic acid

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Introduction

One of the most important research topics in regenerative medicine is the identification of differentiation mechanisms of different types of stem cells, especially differentiation into the germ line. The results of various studies led to design appropriate methods for differentiating the stem cells into male germ cells that provide a promising approach in the treatment of infertility (1-3). Among various sources of stem cells, the umbilical cord is considered as an interesting and beneficial source (4). Some of these benefits include having greater amounts of cells compared with bone marrow; having lower rate of graft-versus-host-disease; no special ethical problem; ease of collection (because umbilical cord is a waste tissue during post-partum); having lower immunological issues rather than embryonic stem cells; it is painless for mother and child; and it reduces the risk of infectious diseases transmission, especially Epstein-Barr virus and cytomegalovirus (5-7). Today, it has been known that human umbilical cord mesenchymal stem cells (hUMSCs) possess some properties of both embryonic and adult

stem cells (8). Therefore, their differentiation potential is higher than MSCs, including bone marrow stem cells (9). Infertility, as one of the most common problems among couples, affects about 10%–15% of males and females. Approximately, half of these cases are associated with males, many of whom suffer from oligospermia or azoospermia. Other factors that affect male infertility include increased apoptosis, stress, and excessive use of glucocorticoids (10,11). For infertility treatment, extensive research and therapeutic methods such as hormone therapy, in vitro fertilization, intracytoplasmic sperm injection, embryo, and ovule or sperm cryopreservation have been carried out (12). In recent decades, the scientists used different sources of stem cells such as embryonic stem cells, bone marrow stem cells, pancreatic stem cells, and stem cells derived from fetal porcine skin for in vitro differentiation toward germ cells. The ethical issues related to the use of embryonic stem cells and the probability of various tumors generation have caused scientists to focus on adult stem cells (2,3). For the first time in 2006, the differentiation of mouse bone marrow MSCs into male germ cells was

demonstrated in the presence of retinoic acid (12). Retinoic acid is an oxidant form of vitamin A, which can differentiate embryonic stem cells into progenitor germ cells (13,14). The present study aimed to investigate the potential of isolated mesenchymal stem cells (MSCs) by modulated explant/enzyme method (MEEM) method, as previously published (15), in expressing germ cell markers and changing morphology.

Materials and Methods

Derivation and expansion of MSCs from human umbilical cord

The isolation and expansion of hUMSCs was according to the method previously published (15). Briefly, fresh umbilical cords were conveyed to sterile phosphate-buffered saline (PBS) and rinsed to remove any vestige of blood. After preparation stages such as cutting of tissue (10 cm), the vessels were detached and shut in the terminal region with a sterile plastic clamp. Vascular loops and tissue fragments were partially digested by MEEM method (15). Upon incubation of explants in collagenase type I (for 3 hours) and inactivation of collagenase by Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, Life Technologies, US) containing 10% fetal bovine serum (FBS, Life Technologies, US), vascular loops and partially digested explants were cultured in DMEM-LG. When the confluency of cells reached 80%–90%, they were spilt at 1:3 ratio for the next experiments.

Multilineage differentiation and cell surface analysis

For identification of surface antigens, the hUMSCs (1×10^6) were stained with anti-CD105, anti-CD90, anti-CD73, anti-CD44, anti-CD45, anti-CD29, anti-CD34, anti-CD31, and HLA-DR (Abcam, UK). The related isotype controls including FITC- and PE-conjugated mouse IgG isotype antibodies were used for negative staining. Following the incubation at room temperature in the dark (20 minutes), stained cells were resuspended in 500 μ L PBS and analyzed by CyFlow[®] Space flow cytometer (Partec, Germany). The histograms were generated based on computed results using Windows[™]-based flow cytometry software.

Preparation and incubation with differentiation medium

Differentiation medium containing 10^{-5} M retinoic acid, 1 mM testosterone, 5% FBS, and 50% conditioned media were prepared. Conditioned media obtained from testes of 5- to 7-day-old Syrian male mice. Briefly, after killing, testicles of mice were isolated and triturated, then centrifuged at 1000 rpm for 10 minutes. In the next step, supernatant was discarded and the cells were cultured. The conditioned media were gathered after 10 days and every 3 days afterward. The conditioned media were centrifuged, filtered (0.22- μ m filter), and stored at -20° C. The medium of hUMSCs monolayer was completely removed and replaced with the differentiation medium as described above. The half of old differentiation medium was replaced with a new differentiation medium every day. Also, the morphology of cells was monitored daily.

RNA isolation, cDNA synthesis, and RT-PCR

The expression of DDX4, DAZL, and PIWIL2 was investigated in comparison to the positive and negative controls using reverse transcription polymerase chain reaction (RT-PCR). Total RNA was purified and eluted in 10 μ L of RNase free water. Then, 1 μ g of total RNA was subjected to cDNA synthesis (Yektatajhezma [YTA], Tehran, Iran) and transferred into the RT-PCR reaction. The gene-specific primers are detailed in Table 1. The amplified products were separated on 2% agarose gel.

Western blot

The cells (2×10^6) were harvested, rinsed with PBS, and extract prepared with lysis buffer (10% Glycerol, 10% SDS, 25% β -mercaptoethanol, Tris-HCl 0.5M pH 6.8, and bromophenol blue 0.5%) containing 1% protease inhibitors. Samples were placed on ice for 60 minutes. Protein concentrations of the cell lysate were quantified by the Bradford assay. The proteins were transferred from SDS-PAGE to a PVDF membrane (16 h/86 mA and 2 h/200 mA) in a transfer buffer (Tris 25 mM, glycine 192 mM, methanol 20%). DDX4, DAZL, and PIWIL2 were detected using primary polyclonal anti-DDX4 Ab, anti-DAZL Ab, and anti-PIWIL2 Ab (Abcam, USA) at a dilution of 1:2000 in blocking solution 5% BSA in wash

Table 1. Gene-specific primer sequences

Gene	Sequence	Annealing	Size of product
c-kit	F:CGTTCTGCTCCTACTGCTTCG R:CCCACGCGGACTATTAAGTCT	60	117
Oct3/4	F: GGAAGGTATTGAGCCAAACGACCATC R: CCAGTTGCCTCTCACTCGGTTCT	61	209
DDX4	F: TCATACTTGCAGGACGAGATTTG R: AACGACTGGCAGTTATCCATC	53	117
DAZL	F: ATGTTGTACTCCGGCTTATCA R: CCATTTCCAGAGGGTGGAGTA	58	118
PIWIL2	F: CTGAAACGGGAAATGCTTCCA R: CCTGTCCTTGCCTACCAGATTA	60	78
GAPDH	F: GAGTCCACTGGCGTCTTCAC R: ATGACGAACATGGGGGCATC	60	110

buffer (TBST) at 4° overnight followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody at 37°C for 60 minutes and developed with a Western blotting Luminol Reagent (Thermo Fisher Scientific, USA). The bands were visualized by imaging using the LI-COR Odyssey[®] scanner and densitometry was accomplished using LI-COR Biosciences software.

Results

Confirmation of MSCs properties

At first, the cells were seeded in six-well plates (2×10^4 cells /well) with complete media to reach 90% confluency. To survey adipogenic differentiation, cells were stained with 0.3% Oil red O solution (Sigma, MI) for 30 minutes at room temperature. For osteogenic induction, cells were stained with Alizarin red S (Fluka, Buchs SG, Switzerland). To evaluate the expression of some major genes associated with the mesenchymal phenotype, the expression of four markers including CD105, CD90, CD73, and CD44 was confirmed. Also, no expression of hematopoietic stem cell markers such as CD45, CD34, and endothelial cell marker CD31 or HLA-DR verified (Figure 1).

Morphological alteration followed by treatment

The morphology of treated cells was observed every day. After 24 hours, the first morphological changes appeared. After 48 hours, severe morphological changes including rounding, elongation, and narrowing were generated on one side of the cytoplasm. At 72 hours, the morphological changes resulted in the formation of cells with a long cytoplasm and central rounded (Figure 2).

Expression of germ cell-specific markers in mRNA and protein levels

At day 3 after treatment with conditioned media as described above, the expression of C-KIT, DAZL, PIWIL2, DDX4, and OCT3/4 in treated and untreated hUMSCs was detected by RT-PCR or Western blot analysis. The result

of RT-PCR showed the high expression level of C-KIT, DAZL, PIWIL2, and DDX4 mRNA in treated hUMSCs compared to negative controls (Figure 3). However, the expression level of OCT3/4 decreased. The expression alteration of DAZL, PIWIL2, and DDX4 verified using Western blot at the protein level (Figure 4).

Discussion

According to our results, it seems that hUMSCs may be able to differentiate into precursors of the male germ cells in the presence of combined effects of retinoic acid, testosterone, and conditioned media within 3 days. Also, some germ cell markers including C-KIT, DAZL, PIWIL2, and DDX4 can up-regulate under conditions as mentioned above. Nayernia et al showed that during 14 to 21 days, bone marrow MSCs can differentiate into gonadal precursors. In our study, decreasing the differentiation time to 3 days may be due to the source of MSCs or concentration of retinoic acid, which has been used in many previous studies as 10^{-6} M; but in our study, the concentration was 10^{-5} M. Undoubtedly, further investigation should be required in this respect. Also, additional evaluations should be well-planned for determining the precise step of differentiation (16). Generating human germ-like cells from some MSCs (e.g., bone marrow MSCs and umbilical or Wharton's jelly-derived MSCs) is largely documented (17-21), and the efficacy of the combined use of retinoic acid and gonadal conditioned media has already been known (22-24). In this research, we compared the potential of isolated MSCs by MEEM method for the expression of germ cell markers and changing the morphology with previous study. According to the literature, the expression of DDX4 is restricted to the germ line. Typically, DDX4 is expressed in human during the early stages of migration; following a decrease in the spermatogenesis stage, it has a poor to moderate expression in the spermatogonial stage. Finally, in spermatocytes and spermatids, the expression of DDX4 is greatly increased, but not in spermatozoa (25).

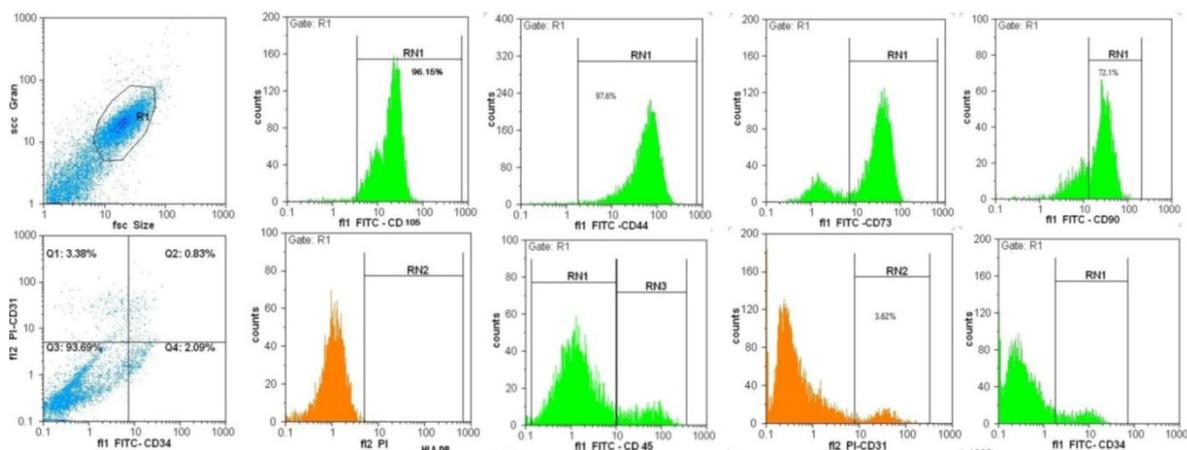


Figure1. Flow cytometry-based immunophenotyping of umbilical cord mesenchymal stem cells derived by MEEM. Cells are prominently positive for CD105, CD44, CD73, and CD90 and negative for HLA-DR, hematopoietic surface cell markers CD45, CD34, and endothelial cell surface antigen CD31.

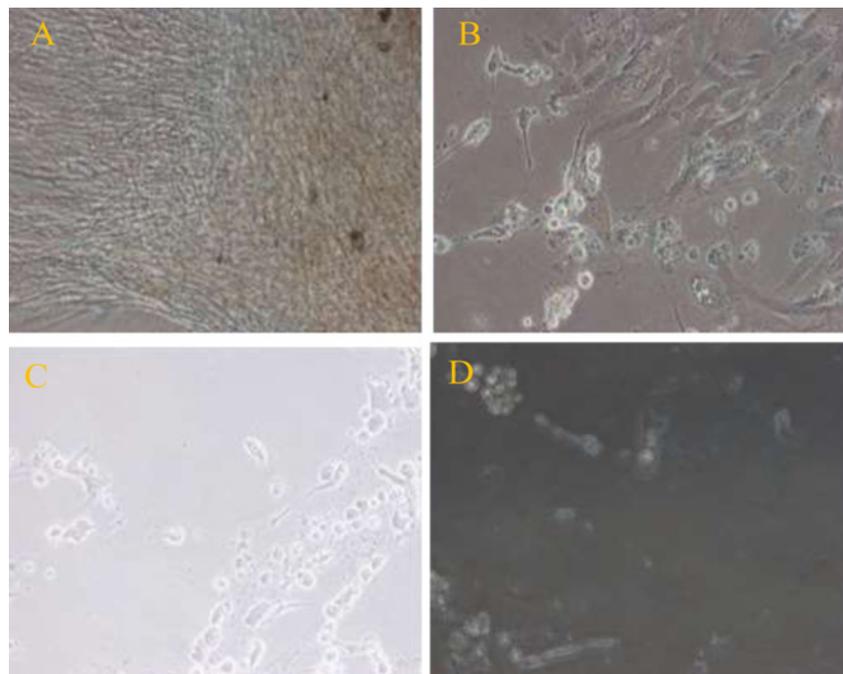


Figure 2. Morphological changes of hUMSCs following treatment. Untreated hUMSCs (A). The morphological changes began after 24 h including narrowing on one side of the cells (B). The morphological changes after 48 h are very severe (C). Severe morphological changes including rounding, elongation and narrowing on one side of the cytoplasm were seen in most of the cells, after 72 h (D).

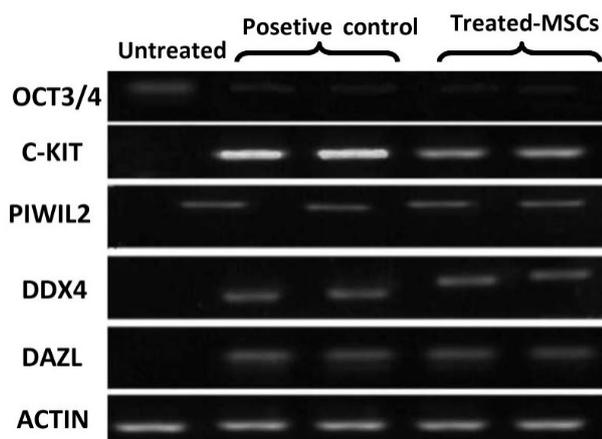


Figure 3. RT-PCR analysis of DAZL, PIWIL2, DDX4, C-KIT, and OCT3/4. Positive control: Human testicular tissue. Treated-MSCs: Treated hUMSCs after 72 h.

In our study, DDX4 expression was confirmed at the level of protein and RNA. However, the expression of PIWIL2 in primary spermatocytes is partially, but it significantly increased in spermatids (26). Also, DAZL is initially expressed in the primary germ cells, then continued during spermatogenesis (27). Since doubling time of bone marrow stromal cells is different from hUMSCs (96 hours vs. 36 hours), the potential of differentiation of them into the germ line may also be different (28). Our results indicated the upregulation of specific genes including DAZL, PIWIL2, and DDX4, which confirmed the differentiation of hUMSCs toward germ cells. Downregulated expression of OCT3/4 (as a stemness gene) determined the transferring from stem cell toward next

differentiation stages. On the other hand, the upregulated expression of C-KIT represents the differentiated MSCs. Previously, human and mouse embryonic stem cells, bone marrow, or pancreas were used to differentiate into the germ cell (12 29 30). Recently, the use of MSCs in regeneration medicine has increased in various studies due to the clinical importance and numerous advantages. Growing evidence suggests that MSCs may be a suitable candidate for in vitro germ cell generation (31-33). In this regard, we reported that isolated hUMSCs by MEEM method might be an easily obtained source to derive germ cells; however, further information and research would be required to support this claim.

Conclusion

The results of current study showed that our method

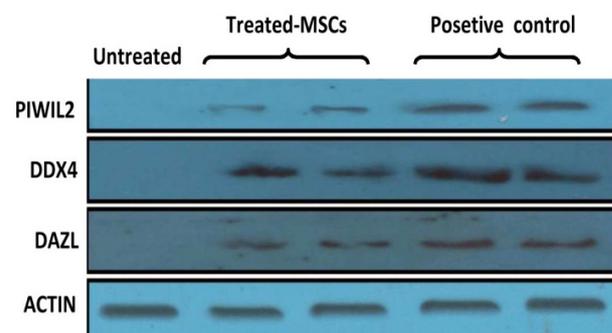


Figure 4. Investigation of PIWIL2, DDX4, and DAZL expression by western blotting after 72 h. Positive Control: Human testicular tissue. Treated-MSCs: Treated hUMSCs after 72 h. Actin was used as a control.

of isolation had no adverse effects on differentiation potential of hUMSCs.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The study protocol was approved by the Research Ethics Committee of Shahrekord University of Medical Sciences (SKUMS), which was according to the Helsinki Declaration (IR.SKUMS.REC.1394.249).

Authors' Contribution

MBD designed and directed the study. AS, AMG, and SR carried out the experiments and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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