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Inductive Role of Sustentacular Cells (Sertoli Cells) Conditioned Medium on Bone Marrow Derived Mesenchymal Stem Cells

Papel Inductivo del Medio Acondicionado de Células Sustentaculares (Células de Sertoli) en las Células Madre Mesenquimales Derivadas de la Médula Ósea

Mahdieh Hajian Monfared[1]; Mohammad Akbari[1]; Iraj Ragerdi kashani[1]; Somayeh Solhjoo[1]; Heidar Tooli[1]; Ameneh Omidi[2]; Freshteh Aliakbari[1]; Sahar Ijaz[1]; Tahmineh Mokhtari[1] & Tayebeh Rastegar[1]

HAJIAN MONFARED, M.; AKBARI, M.; KASHANI, I. R.; SOLHJOO, S.; TOOLI, H.; OMIDI, A.; ALIAKBARI, F.; IJAZ, S.; MOKHTARI, T. & RASTEGAR, T. Inductive role of sustentacular cells (Sertoli cells) conditioned medium on bone marrow derived mesenchymal stem cells. *Int. J. Morphol., 35(4)*:1597-1606, 2017.

SUMMARY: Male germ line stem cells are key factors for male spermatogenesis and fertility. Sustentacular cells (Sertoli cells) as somatic cells play a pivotal role in creating essential microenvironment for the self-renewal and differentiation of the male germ line cells. Mesenchymal stem cells are recognized as self-renewing and multipotent cells able to differentiate into multiple cell types. The generation of male germ cells from mesenchymal stem cells may provide a therapeutic method to treat male infertility. In this study, Bone marrow derived mesenchymal cells (BMMSCs) were retrieved from the bone marrow of 6-8-week old Naval Medical Research Institute (NMRI) mice. Sustentacular cells (Sertoli cells) were isolated and made rich using lectin coated plates. Sustentacular cell condition medium (SCCM) was collected after different time intervals. Then the BMMSCs were cultured with different concentration of SCCM and Dulbecco's Modified Eagle's medium (DMEM) at various times. Specific markers of Germ line cells were evaluated by using Reverse transcriptase polymerase chain reaction (RT-PCR) and immunocytochemistry. The results showed that BMMSCs cultured with SCCM for 48h exhibited germ line specific transcripts (Mvh, Iid4, piwil2) ($p< 0.05$) and markers (Mvh, Scp3). Our findings represent that culturing BMMSCs with SCCM may lead to effective differentiation of BMMSCs into germline cells and provide a treatment strategy for male infertility.

KEY WORDS: Sustentacular cells conditioned medium; Bone marrow derived Mesenchymal stem cells; Differentiation; Culture.

INTRODUCTION

Infertility is an important issue among the problems of developed societies. About half of the cases of infertility are related to male infertility. Although there are many treatment methods for infertility such as IVF or ICSI but these methods are not effective in some cases with defective spermatogenesis (Newson & Smajdor, 2005; Valsangkar *et al*., 2011; Shirazi *et al*., 2012). Various studies have been done on derivation of male germ cells from stem cells. Bone marrow mesenchymal stem cells (BMMSC) are a good source for derived germ cells but these derived cells do not have a potential of spontaneous differentiation (Nayernia *et al*., 2004; Hua *et al*., 2009). Therefore, inductive factors are necessary for the differentiation of these derived cells.

Sustentacular cells are known as nurse or mother cells for Spermatogonial stem cells (SSCs) in testis. They encompass SSCs and regulate their differentiation by secreting growth factors (Mohamamadi *et al*., 2010; Chui *et al*., 2011). They act as regulators of spermatogenesis (Kissinger *et al*., 1982; Sofikitis *et al*., 2005). The presence of sustentacular cells with high amounts of Follicle stimulating hormone (FSH) and testosterone progressed meiotic division, reduced apoptosis and enhanced survival of germ cells in cultured human testis tissue or cell suspensions (Tesarik *et al*., 1998; Tesarik *et al*., 2001). Likewise, many studies have demonstrated that co-culture with Sustentacular cells and hormone enforces post-meiotic progression *in vitro* (Lee *et al*., 2001; Sousa *et al*., 2002; Lee *et al*., 2006a).

Recent studies have illustrated that mesenchymal stem cells could differentiate into germ cells under suitable conditions. Testicular-cell-conditioned medium induced Human Umbilical Mesenchymal Stem Cells (hUMSCs) to differentiate into germ cells (Huang *et al*., 2010). The results

[1] Anatomy Department, School of Medicine, Tehran University of Medical Science, Tehran, Iran.
[2] Anatomy Department, School of Medicine, Tarbiat Modares University, Tehran, Iran.

showed that secretory products derived from Sustentacular cell conditioned medium increased cell proliferation and enhanced dopaminergic neuronal differentiation of the 796RMB cell line (Shamekh *et al*., 2008). Sustentacular cell condition medium can significantly differentiate the human embryonic stem cell (hESC) lines into the germ cell lineage (Geens *et al*. 2011). Conditioned medium collected from testicular cell cultures developed Embryonic Stem Cells (ESCs) into ovarian structures containing oocytes (Lacham-Kaplan *et al*., 2006).

Today, testicular somatic cell culture has become an important and innovative tool for applied research in reproductive biology (Langenstroth *et al*., 2014). Development of in vitro differentiation and culture media would help to introduce a process of inducing spermatogenesis (Sisakhtnezhad *et al*., 2015). Therefore, we extracted condition medium from adult mouse Sustentacular cells at different time intervals to evaluate its effect on the induction of BMMSCs to express male germ cell markers and differentiate into male germ line cells.

MATERIAL AND METHOD

Experimental animals: Six to-eight-week-old NMRI male mice were kept under suitable conditions with free access to food and water. The ethics committee of Tehran University of Medical Sciences approved for this animal experiment in concurrence with University guidelines.

BMMSCs Isolation and culture. BMMSCs were collected from 6-8 week old NMRI mice by flushing method of aspiration. After centrifuging, suspended cells were plated in DMEM (Gibco, Germany) enriched with 15 % FBS (Gibco, Germany), 100 u/ml Penicillin and 100 mg/ml streptomycin (Gibco, Germany). Then the cells were incubated at 37 °C with 5 % CO2. The medium was replaced every 3 days until it reached enough confluence. After 3 passages the mesenchymal entity of BMMSCs was proved with special markers (expression of CD44 and CD73 and non-expression of CD45 and CD11b) using flow cytometry and their multi-potential entity was proved by their differentiation into osteogenic and adipogenic cells within 21 days (Soleimani & Nadri, 2009).

BMMSCs Pluripotency. The cells were obtained from the third passage cultured in osteogenic and adipogenic medium. Osteogenic medium consisted of DMEM enriched with 10mg/ml ascorbic 2-phosphate (Sigma, USA), 10nM Dexamethasone (Sigma, USA), 10 mM B-Glicerol phosphate (Sigma, USA). Adipogenic medium consisted of DNEM enriched with 50 mg/ml Ascorbic 2 phosphate (Sigma, USA), 50 mg/ml Indomethacin (Sigma, USA), 100 nM Dexamethasone (Sigma, USA). The conditioned media were incubated in 95 % humidified and 5 % CO_2 atmosphere at 37 °C. After 3 weeks, we evaluated the cells with Alizarin red staining and Oil red staining for Osteogenic cells and Adipogenic cells respectively (Hosseinzadeh Shirzeily *et al*., 2013).

Flow Cytometry. For the demonstration of mesenchymal stem cells obtained from bone marrow, superficial specific markers of BMMSCs were analyzed by flow cytometry technique according to the manufacturer's instructions. The cultured cells after third passage were harvested by trypsin (Invitrogen, USA) and finally 10^6 cells were used for analysis. Flow cytometric assay was performed and mesenchymal stem cells CD markers were recognized and then Win MDI 2.9 software was used for the analysis. CD44 (12-0441-81, eBioscience, USA) and CD73 (550257, BD-Bioscience, USA) were considered as mesenchymal stem cells markers and, CD45 (341071, BDbioscience, USA) and CD11b (110112-41, eBioscience, USA) as hematopoietic and macrophage markers. The cells were incubated in 5 mg/ml fluorescein isothiocyanate (FITC) conjugated antibodies. FITC mouse IgG2A (11-4724, eBioscience, UK) and Rat IgG1 (ab18412, abcam, USA) were used as isotype controls (Mazaheri *et al*., 2011; Rastegar *et al*., 2013).

Sustentacular cells isolation. Sustentacular cells were isolated from the adult mice testis (6-8 weeks old) as formerly described by Scarpino *et al*. (1998). In brief, culture dishes were coated with 5 mg/ml of Datura Stramonium Agglutinin

Table I. Primer sets used for amplification of genes.

Genes	Primers sequences (5' to 3')	Product length (bp)
Mvh	Forward primer: GAGGGGGAAGAGGCAGTTTC	648 bp
	Reverse primer: TGGTAAGTGTCACCATTGCCT	
ID4	Forward primer: AAACAAGCCACCGGAGGAAA	166 bp
	Reverse primer: AGCAAAAGCTCTGCAAGGGA	
PIWIL2	Forward primer: GGGCTGGAATAGGAGGGAAA	125 bp
	Reverse primer: CTGTGTCAAGACCCATGCCA	
Beta actin	Forward primer: CTGGCCTCACTGTCCACCTT	115 bp
	Reverse primer: AAACGCAGCTCAGTAACAGTCC	

(DSA; Sigma) lectin in PBS at 37 °C for 1 h. The coated plastic dishes were then washed thrice with 0.5 % Bovine Serum Albumin (BSA; Sigma). The testis cell suspension obtained by enzymatic digestion was placed on lectin-coated dishes and incubated for 1 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After incubation, the floating cells were collected by washing them twice with DMEM medium. After that, DMEM and 10 % FBS were added. Seven days later Sustentacular cells formed a confluent layer.

Preparation of condition medium. When the Sustentacular cells formed a confluent layer, the condition medium was collected after 24 and 48 h and centrifuged at 1000g for 10 minutes. The supernatant was used as culture medium (Shamekh *et al*.; Geens *et al*.).

Immunocytochemistry for characterization of adult Sustentacular cells. Sustentacular cells expressed vimentin in their cytoplasm and this property was detected in their culture by immunocytochemistry. After their fixation with 4 % paraformaldehyde, permeabilization by 0.4 % Triton X100 (Sigma) and blocking with 10 % goat serum (Sigma), the cells were incubated for 2 h at 37 °C with mouse monoclonal anti-vimentin antibodies in 1:100 (Sigma) dilution. The cells were then, washed with Phosphate Buffer Solution (PBS) and incubated for 3 h with goat anti-mouse FITC conjugated antibodies, the secondary antibodies, in dilution of 1:100 (Sigma). Control cells were treated under equal conditions except for the removal of the first antibody. Nuclei were stained with 5 mg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) (Anway *et al*., 2003).

Induction of BMMSCs to germ like cells. For this purpose, the BMMSCs obtained from third passage were used. The cells were cultured in 4 different culture media that are:1) 50 % SCCM obtained after 24 h + 50 % DMEM, 2) 100 % SCCM obtained after 24 h, 3) 50 % SCCM obtained after 48 h+50 % DMEM and 4) 100 % SCCM obtained after 48 h. SCCM obtained after 24 and 48 h from adult mice Sustentacular cells as described above and BMMSCs cultured in SCCM for 1-3 weeks and, the cells cultured in DMEM, Penicillin Streptomycin (Gibco, Germany), FBS 10 % (Gibco, Germany) for 1-3 weeks was considered as control group. Within the culture period, the medium was replaced with fresh medium every 3rd day in all the groups and the cells were incubated at 37 °C in atmosphere of 95 % air and 5 % CO_2 (Geens *et al*.). Finally the cells were evaluated by RT-PCR and Immunochemistry in order to recognize induced cells.

Identification of germ cell by Reverse transcriptase polymerase chain reaction. After two weeks, the expression of Mvh, Piwil2 and Id4 Transcripts was detected using RT-PCR. Primer sequences are given in Table I. Extraction of total RNA was done by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RT-PCR was performed by cDNA synthesis kit (Bioneer, South Korea) using 1 ml of total RNA according to the manufacturer's protocol. PCR was performed using Tag DNA polymerase (Cinagene, Tehran, Iran) in a Gene Amp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After an initial denaturation at 94 °C for 5 min, cDNA was subjected to 33 cycles of PCR. Testicular cells from adult testis were used as positive control and water as the negative control. To normalize RT-PCR samples, expression of b-actin was performed. PCR products were discovered on 2 % agarose gels, and the intensity of bands was assayed by UVI doc gel documentation system (Avebury house 36 a union lane Cambridge CB4 1QB-UK) (Zanganeh, Rastegar *et al*. 2013).

Identification of germ cell by Immunofluorescence. First, the cells were washed twice in PBS and fixed with 4 % paraformaldehyde, and then Triton X-100 was added for penetrating into the cells. In order to prevent expression of non-specific proteins at room temperature BSA was used. In the next step, primary antibodies of Anti Mvh (ab13840, abcam, USA) and Anti Scp3 (ab150292, abcam, USA) were added separately at the end of week 1 and week 3 respectively, and maintained at room temperature for 3 h. The cells were washed with PBS and after the addition of secondary antibodies (goat anti-mouse) labeled with FITC, incubated for 2 h and preserved in dark room. Later, the antibodies were thrown away and the cells were washed. Control cells were treated under equal conditions except for the removal of the first antibody. For demonstrating the nuclei, DAPI was added. Finally, the differentiated cells were detected by fluorescent microscope (Baazm *et al*. 2013).

Statistical analysis. The results were presented as mean ± SD. The statistical significance between the mean values was determined by one-way analysis of variance (ANOVA). A level of $P \leq 0.05$ was considered as significant value.

RESULTS

BMMSCs culture, Pluripotency and Identification. Morphological characteristics of the cultured mouse BMMSCs were similar to others. On Day 1, most of the cells were still mononuclear (Fig. 1A). On Day 2, some spindle shaped cells appeared among the mononuclear cells (Fig. 1B). The results revealed that spindle shaped BMMSCs repetition reached as much as 80-90 % confluence within 5 and 8 days respectively (Fig. 1C). Fibroblast-like cells grew out and the cells were passaged on Day 7.

The findings that demonstrated that the cells differentiated to osteogenic and adipogenic cells were illustrated with Alizarin red and Oil red staining respectively. Oil-red staining showed lipid-rich vacuoles formation in the mouse BMMSCs after 3 weeks of adipogenic induction (Fig. 2A). Alizarin red staining demonstrated that mineralized nodules formed in the BMMCs after 3 weeks under the osteogenic induction (Fig. 2B).

Moreover, BMMSCs significantly expressed the mesenchymal superficial markers of CD44 and CD73, and did not express non- mesenchymal superficial markers of CD45 and CD11b (Fig. 3). Therefore, these cells had mesenchymal and pluripotent cell characteristics.

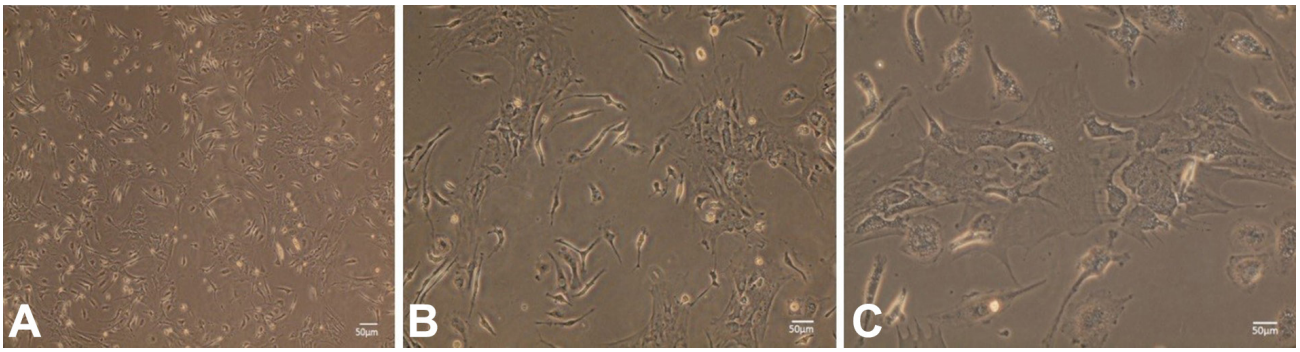

Fig. 1. Microscopic morphology of Mesenchymal stem cells derived from 4-6 week-old male mice after 3 passages. On Day 1, most of the cells were still mononuclear (A). On Day 2, some spindle-shaped cells appeared among the mononuclear cells (B). On Day 7, cells reached 100 % confluence and had uniformfibroblast-like morphology (C). Scale bar=50μm

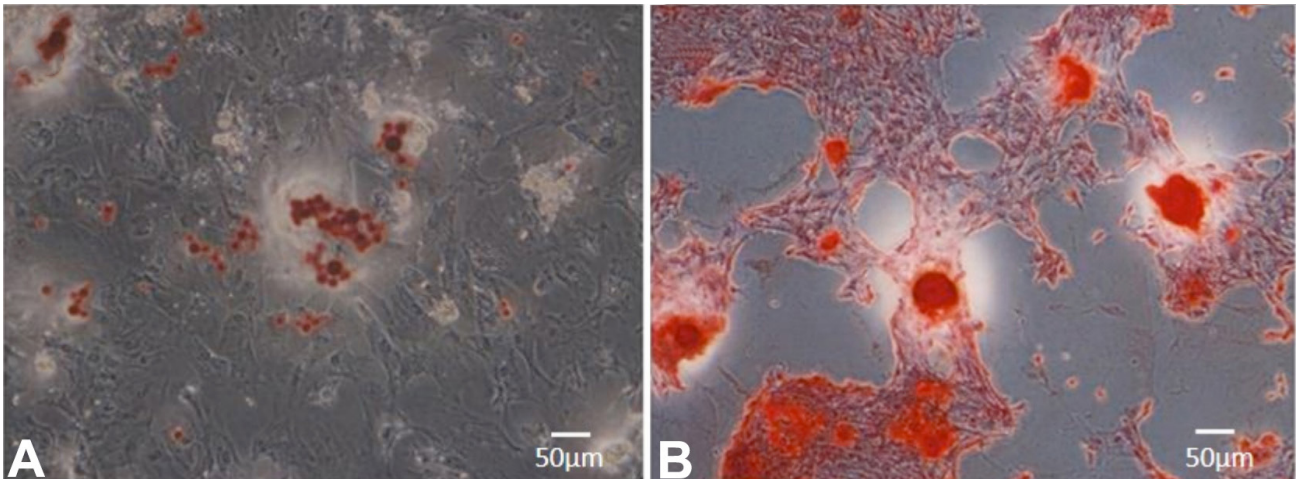

Fig. 2. Microscopic morphology of Mesenchymal stem cells derived from 6-8 week-old male that have been induced to Adipocyte and Osteocyte: A-The accumulation of Lipid droplets with oil red coloration is the sign of differentiation to Osteocyte. B- The calcium deposits with Alizarin red coloration is the sign of differentiation to Adipocyte. Scale bar=50 μm

Sustentacular Cell Isolation and Characterization. Sustentacular Cells cultured on lectin DSA coated plates were initially round and firmly adherent to the bottom of the dish. After 1 day, cells began to flatten, spread out and take on an epithelioid appearance (Fig. 4A). The presence of Vimentin protein on Sustentacular cells was confirmed by immunocytochemistry, demonstrating that the majority (up to 85 %) (Data not shown) of freshly isolated cells attached to lectin were Vimentin positive (Fig. 4B). All these results indicated the Sustentacular cells were properly isolated (Figs. 4B and C).

BMMSCs Differentiation. Isolated BMMSCs were cultured in DMEM enriched with 15 % FBS, 100 n/ml Penicillin and 100 mg/ml streptomycin (Gibco, Germany). In order to provide the optimum cell culture condition for differentiation of BMMSCs to germ like cells, we then compared four different conditions; in 50 % SCCM with 50 % DMEM or In SCCM only, both for 24h and 48h. After co-culture with Sustentacular cells condition medium, BMMSCs demonstrated good viability in terms of morphological appearance. The cells exhibited expression levels of Mvh, Id4 and Piwil2 transcripts. A significant ($p<0.05$) rise in their cDNA levels

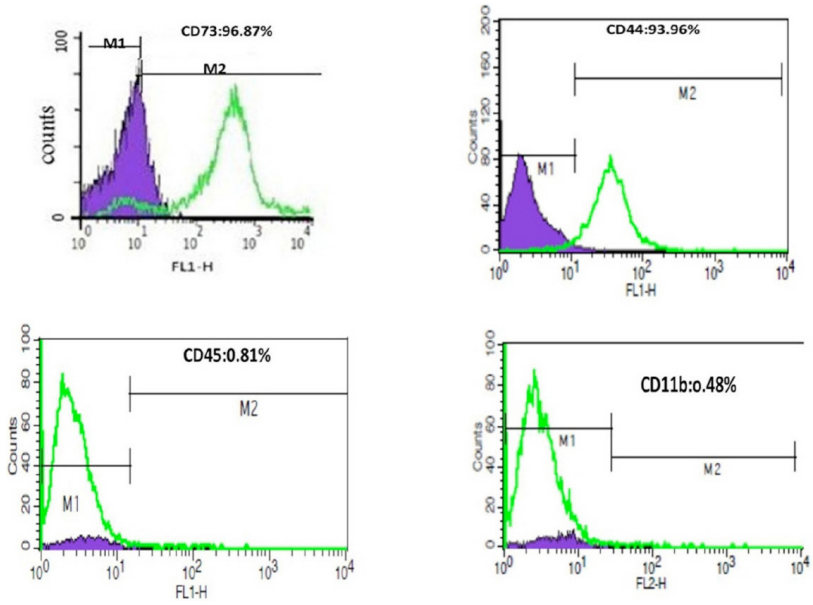

Fig. 3. Flow cytometric analysis for detection of superficial markers of mesenchymal stem cells (CD44 and CD73) (upper), Expression of superficial markers of hematopoietic and macrophage cells (CD45 and CD11b) (lower).

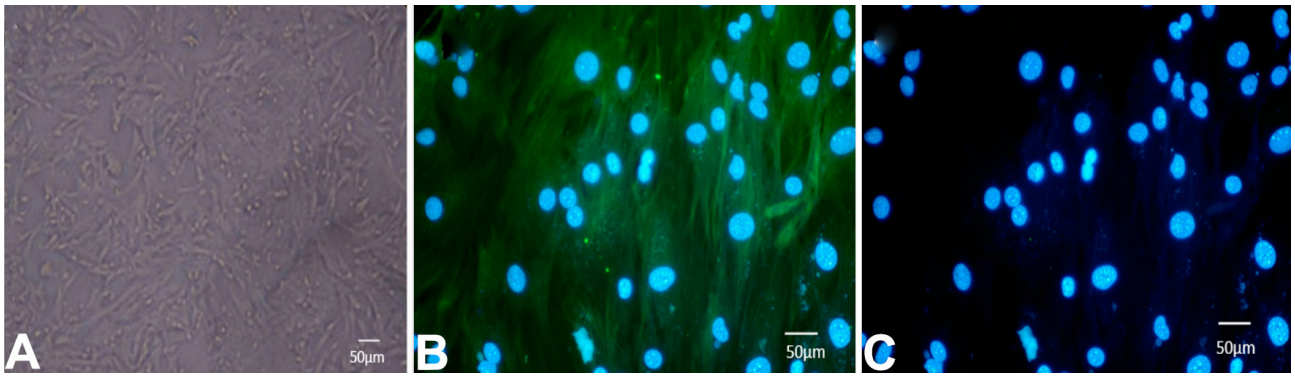

Fig. 4. Microscopic morphology of Sustentacular cells derived from 6-8 week-old male mice. Sustentacular cells were isolated using lectin DSA and characterized by immunocytochemistry. Monolayer Sustentacular cells began to flatten and spread out following one day plating on lectin DSA. A-Sustentacular cells. B- Sustentacular cells were positive for Vimentin in cytoplasm (green color) and nuclei were stained with DAPI (blue color). Scale bar=50 μm

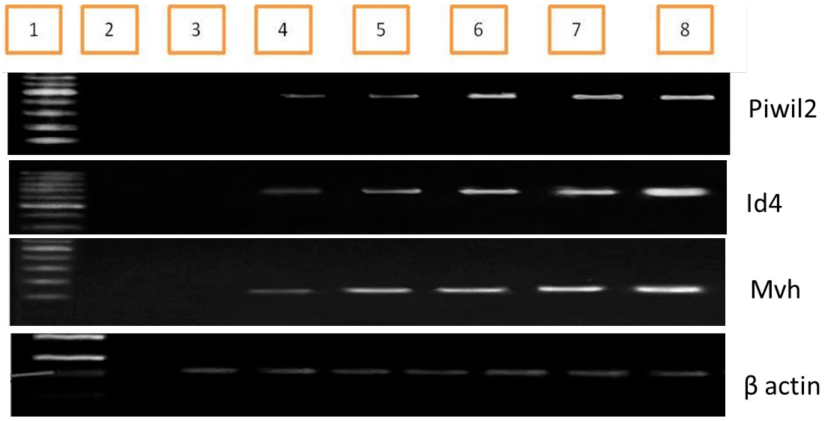

Fig. 5. Electrophoresis of PCR products on 2 % agarose gels. After 7 days of cultivating of mesenchymal stem cell in different conditions medium, the expression of some specific transcripts were studied by RT-PCR. 1-100bp DNA Ladder 2- negative control of RT-PCR(H2O) 3-negative control 4- Sustentacular cells condition medium after 24 h (50 %) 5- Sustentacular cell condition medium after 24 h (100 %) 6- Sustentacular cell condition medium after 48 h(50 %) 7- Sustentacular cell condition medium after 48 h(100 %) 8-positive control (testicular extraction).

was detectable in the groups cultured for 48 hrs with SCCM only (Figs. 5, 6).

After exhibiting expression levels of Mvh transcripts, the presence of Mvh and also Scp3 proteins were confirmed by immunocytochemistry (Fig. 7).

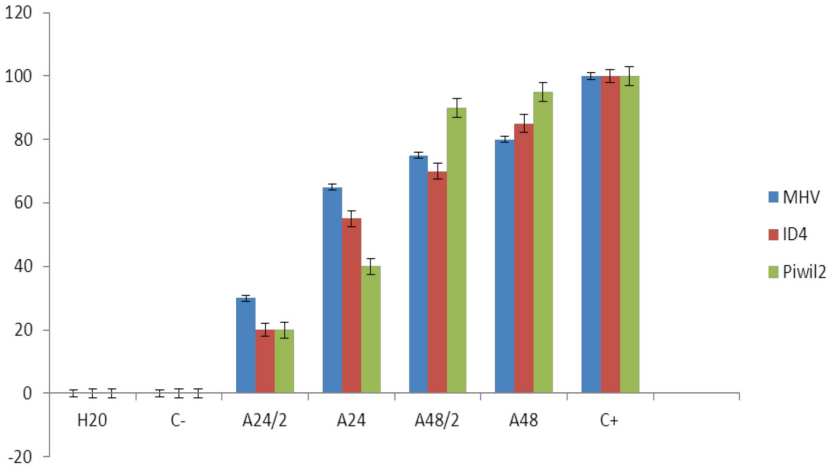

Fig. 6. Ratio of Genes/β-actin has been shown by a histogram in different groups ($p \leq 0.05$). H_2O, water and C-, liver as negative controls; A24/2, 50 % Sustentacular cell condition medium after 24 h; A24, 100 % Sustentacular cell condition medium after 24h; A48/2, 50 % Sustentacular cell condition medium after 48 h; A48, 100 % Sustentacular cell condition medium after 48 h and C+, positive control (testicular extraction).

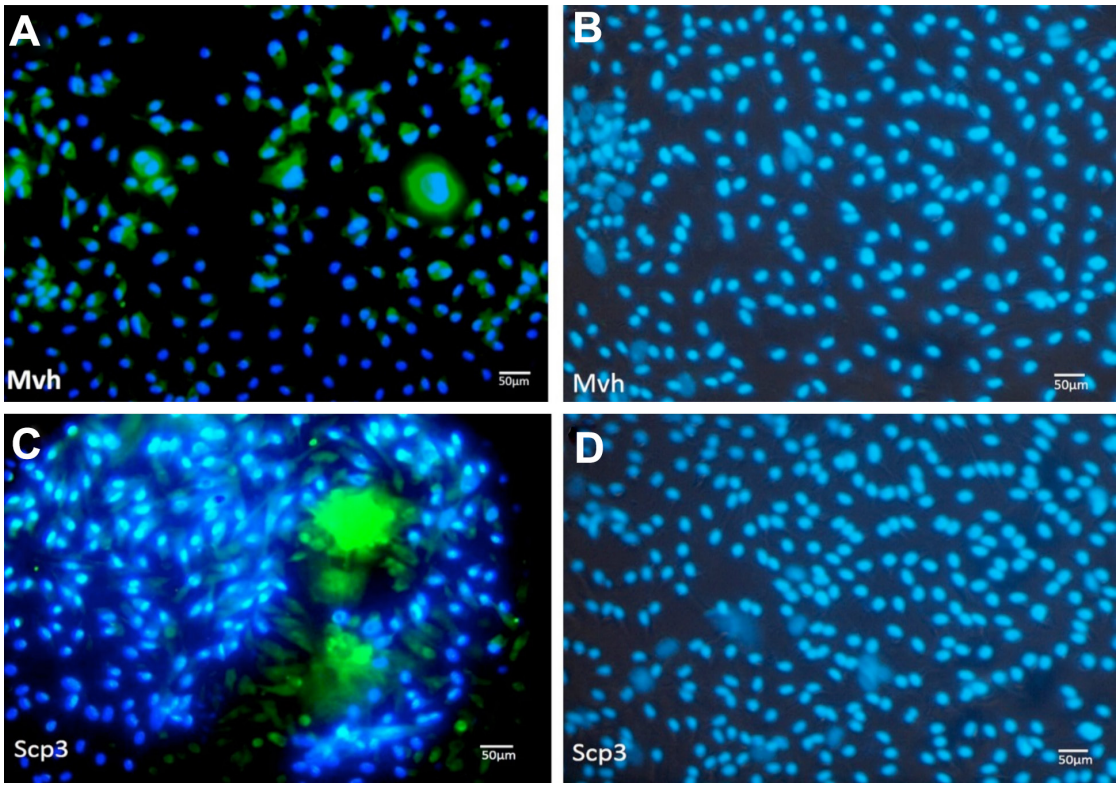

Fig. 7. Microscopic morphology of differentiated male germ like cells derived from BMMSCs of 6-8 week-old male mice that cultured with SCCM by immunocytochemistry for Mvh and Scp3. (A,C) Differentiated BMMSCs that cultured with Sustentacular cell condition medium were positive for Mvh (after 1 week) and Scp3 (after 3 weeks) (green color) and nuclei were stained with DAPI (blue color). (B, D) BMMSCs cultured with DMEM were negative for Mvh.Scale bar=50 μm

DISCUSSION

We demonstrated that the adult Mesenchyme stem cells (MSCs) can differentiate to male germ-like cells. MSCs have been investigated as promising candidates for use in new cell-based therapeutic strategies. MSCs are easily isolated from adult tissues and are not ethically restricted. For five decades, many researchers have been trying to generate male germ cells from several types of stem cells in order to provide therapeutic strategies for male infertility (Hou *et al*., 2014). MSCs derived from bone marrow are a good alternative of adult stem cells that can form a variety of cell types such as fat cells, cartilage, bone (Hua *et al*.). We obtained MSCs from bone marrow and confirmed their multi-potential properties with osteoblast and adipocyte condition medium and by using Alizarin red S and Oil red staining.

MSCs are generally known to be positive for cluster of differentiation, CD73/CD44 as mesenchymal markers and negative for CD45/CD11b as hematopoietic and macrophage markers. They are usually obtained from mesenchymal tissues simply by collecting adherent cells, and are thus heterogeneous. The main problem in the basic research of MSCs is that most analyses have been performed using the heterogeneous bulk MSCs rather than purified populations of single kind of cells, and thus the cells actually responsible for the pluripotent-like phenomenon of MSCs have not been identified (Dezawa, 2016). Mesenchymal entity of the cells was confirmed by flow cytometry. Present study found that superficial markers of CD44 and CD73 were expressed significantly but CD45 and CD11b did not.

These results were in good agreement with that of Domicini *et al*. (2006).

It has been reported that stem cells can differentiate into primordial germ cells and early gametes (Hou *et al*.). Many researchers have used embryonic stem cells to induce germ cells (Hübner *et al*., 2003; Toyooka *et al*., 2003; Clark *et al*., 2004; Geijsen *et al*., 2004) but, they preferred to use adult stem cells because of ethical reasons and tumor genesis related to embryonic stem cells (Brickman & Burdon, 2002). For isolating adult Sustentacular cells, we used Lectin DSA coated dishes, as described earlier by Scarpino *et al*. Isolated cells by this method expressed vimentin in their confirming that these cells were Sustentacular cells (Scarpino *et al*.; Anway *et al*.).

Appropriate culture system is a critical and essential step for successful differentiation of male germ cells. Co-culture with Sustentacular cells, gonadal stromal cells, Vero cells and enriched medium with Retinoic acid and testicular extraction are investigated as culture systems that can increase expression of male germ cell markers (Drusenheimer *et al*., 2006; Nayernia *et al*., 2006; Park *et al*., 2009; Zanganeh, Rastegar *et al*., 2013; Hou *et al*.).

Testicular extracts induced human bone marrow stromal cells (hBMSC) to differentiate into male germ cells (Huang *et al*.; Hou *et al*.). Designing the natural niche for cells provides a valuable therapeutic approach to some diseases. Sustentacular cells produce different growth factors essential for self-renewal and differentiation of male germ line cells (Griswold, 1995). Sustentacular cells have fundamental roles in the organization of testis and the development of its cells by providing constructional, immunological, and nutritional support. SCCM contains inductive factors for germ cell differentiation andcould prove a suitable element for in vitro differentiation of potential cells to germ cells. SCCM significantly improved VASA expression in hESC (Geens *et al*.).

Bone marrow stem cells are able to differentiate into male germ line cells as they exhibited the known special markers of germ cells, such as Dazl, Mvh, fragilis, stella, Piwil2, b1- and a6- (Nayernia *et al*., 2006). Human bone marrow stem cells (hBMSC) transdifferentiated into male germ like cells and expressed specific germ cell markers NANOG,OCT4, STELLA and male germ-cell markers such as DAZL, and SCP3 (Drusenheimer *et al*.; Hua *et al*.). Also Hua *et al*. reported the expression of meiotic markers (scp3) using testicular extraction and Retinoic Acid (Hai *et al*., 2014). Meiotic and post-meiotic markers are more reliable markers, but it has been demonstrated that the progression through the meiotic process is still a challenge in the in vitro differentiation of germ cells (Marques-Mari *et al*., 2009). Our work showed expression levels of Mvh, Id4 and Piwil2 genes and presence of Mvh and Scp3 proteins using Sustentacular cells condition medium for 48h.

There are three important basis to believe in the essential effects of Sustentacular cells on spermatogenesis. First, male germ cells can survive and mature with Sustentacular cells in vivo; second, the male germ cells are supported by Sustentacular cells is fixed in certain species; and finally, endocrine and paracrine regulation of spermatogenesis has to be mediated by Sustentacular cells (Hai *et al*.). So in this study, we evaluated the effects of adult Sustentacular cells condition medium on inducing germ cells from BMMSCs. The derivation and differentiation of germ cells from the BMMSC in adult mice SCCM has not been reported in the previous studies.

BMMSCs were cultured for two weeks in the adult Sustentacular cell condition medium that was obtained after 24 h and 48 h. The differentiation rate was evaluated in

different groups as mentioned above. The cells that were cultured in SCCM derived after 48 h expressed significantly more male germ cell markers than those cultured in SCCM derived after 24 h. In order to recognize differentiated cells, Mvh, Id4 and Piwil2 were used as important markers. Mvh is a primary marker which is expressed in germ cells and is not found in other cells. The expression of Mvh is related to proliferated and differentiated germ cells (West *et al*., 2010). Id4 is a marker that is restricted to a single spermatogonium and it is an essential protein for normal spermatogonial stem cell renewal both *in vitro* and *in vivo* (Hermann *et al*., 2011). Piwil2 is one of the markers of spermatogonia that has an essential role in spermatogonial stem cell renewal and producing germ cell (Lee *et al*., 2006b). SCCM contains inductive factors for germ cell differentiation and could represent an element for in vitro differentiation to germ cells (Geens *et al*.). On the other hand, 72 and 120 hour Embryoid Bodies (EBs were also cultured in Testicular conditioned medium and significant morphological changes occurred in them (Lacham-Kaplan *et al*.).

In order to evaluate the cell differentiation into male germ cells, immunocytochemistry was used to detect Mvh and Scp3 germ cell marker proteins. Scp3 is a member of synaptonemal complex protein that is expressed in the initial phase of meiosis (Eddy, 2002). As expected, the cells in control group neither expressed molecular markers (Mvh, Id4, Piwil2) nor the male specific proteins (Mvh, Scp3), but in the other groups, especially in the one cultured with SCCM derived after 48 h, these proteins were expressed. Consistent with our findings, Geens *et al*. evaluated the effect of SCCM on inducing germ cell. They used extracted condition medium from neonate mice Sustentacular cell and performed RT-PCR and Immunocytochemistry for Vasa and demonstrated positive results (Geens *et al*.).

CONCLUSION

Our findings reveal direct evidence that bone marrow cells can differentiate to putative male germ cells. Similar to neonate SCCM, adult SCCM can be used as a motivating factor for induction of male germ cell from somatic stem cells. Bone marrow can be recognized as a potential source of male germ cells that could preserve sperm production and fertility.

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RESUMEN: Las células madre de la línea germinal masculina son factores clave para la espermatogénesis masculina y la fertilidad. Las células sustentaculares (células de Sertoli) como células somáticas juegan un papel fundamental en la creación de un microambiente esencial para la auto-renovación y diferenciación de las células de la línea germinal masculina. Las células madre mesenquimales son reconocidas como células auto-renovables y multipotentes capaces de diferenciarse en múltiples tipos de células. La generación de células germinales masculinas a partir de células madre mesenquimales puede proporcionar un método terapéutico para tratar la infertilidad masculina. En este estudio, las células mesenquimales derivadas de la médula ósea (BMMSCs) se recuperaron de la médula ósea de ratones de 6-8 semanas de edad del Instituto de Investigación Médico Naval (NMRI). En el estudio se aislaron las células sustentaculares y se enrriquecieron usando placas revestidas con lectina. Se obtuvo el medio de condición celular después de diferentes intervalos de tiempo. Posteriormente se cultivaron las BMMSC con diferentes concentraciones de SCCM y medio de Eagle modificado por Dulbecco (DMEM) en diversos momentos. Se evaluaron marcadores específicos de células de línea germinal usando la reacción en cadena de polimerasa transcriptasa inversa (RT-PCR) e inmunocitoquímica. Los resultados mostraron que las BMMSCs cultivadas con SCCM durante 48h exhibieron transcritos específicos de línea germinal (Mvh, Iid4, piwil2) ($p < 0,05$) y marcadores (Mvh, Scp3). Nuestros resultados indican que el cultivo de BMMSCs con SCCM puede conducir a la diferenciación efectiva de BMMSCs en células germinales y proporcionar una estrategia de tratamiento para la infertilidad masculina.

PALABRAS CLAVE: Medio acondicionado con células sustentaculares; Células madre mesenquimales derivadas de médula ósea; Diferenciación; Cultivo.

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Corresponding author:
Tayebeh Rastegar
Department of Anatomy
School of Medicine Science
Tehran University
Tehran
IRAN

Email: trastegar@tums.ac.ir

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