Original Article
https://doi.org/10.5115/acb.22.246
eISSN 2093-3673
Anatomy & Cell Biology acb

Evaluating the effect of conditioned medium from mesenchymal stem cells on differentiation of rat spermatogonial stem cells

Hoda Fazaeli[1], Mohsen Sheykhhasan[1], Naser Kalhor[1], Faezeh Davoodi Asl[1], Mojdeh Hosseinpoor Kashani[2], Azar Sheikholeslami[1]

[1]Department of Mesenchymal Stem Cells, Academic Center for Education, Culture, and Research (ACECR), Qom Branch, Qom, [2]Department of Biology, Faculty of Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran

Abstract: In cancer patients, chemo/radio therapy may cause infertility by damaging the spermatogenesis affecting the self-renewal and differentiation of spermatogonial stem cells (SSCs). *In vitro* differentiation of stem cells especially mesenchymal stem cells (MSCs) into germ cells has recently been proposed as a new strategy for infertility treatment. The aim of this study was to evaluate the proliferation and differentiation of SSCs using their co-culture with Sertoli cells and conditioned medium (CM) from adipose tissue-derived MSCs (AD-MSCs). Testicular tissues were separated from 2–7 days old neonate Wistar Rats and after mechanical and enzymatic digestion, the SSCs and Sertoli cells were isolated and cultured in Dulbecco's modified eagle medium with 10% fetal bovine serum, 1X antibiotic, basic fibroblast growth factor, and glial cell line-derived neurotrophic factor. The cells were treated with the CM from AD-MSCs for 12 days and then the expression level of differentiation-related genes were measured. Also, the expression level of two major spermatogenic markers of *DAZL* and *DDX4* was calculated. *Scp3*, *Dazl*, and *Prm1* were significantly increased after treatment compared to the control group, whereas no significant difference was observed in *Stra8* expression. The immunocytochemistry images showed that *DAZL* and *DDX4* were positive in experimental group comparing with control. Also, western blotting revealed that both *DAZL* and *DDX4* had higher expression in the treated group than the control group, however, no significant difference was observed. In this study, we concluded that the CM obtained from AD-MSCs can be considered as a suitable biological material to induce the differentiation in SSCs.

Key words: Spermatogonial stem cells, Mesenchymal stem cells, Conditioned medium, Differentiation

Received November 30, 2022; 1st Revised January 28, 2023; 2nd Revised May 27, 2023; 3rd Revised June 27, 2023; 4st Revised August 9, 2023; Accepted August 21, 2023

Introduction

Spermatogenesis is a biological process essential for the male germline's continuity by which haploid spermatozoa are produced. Throughout the male's life, spermatogenesis is a continuous and coordinated process of cell proliferation and differentiation that results in the development of unrestricted numbers of spermatozoa [1]. Spermatogonial stem cells (SSCs), which have the remarkable ability to self-renew and produce differentiated daughter cells that will eventually form spermatozoa, are at the heart of this scheme [2]. In seminiferous tubules, SSCs are rare stem cells found in a niche surrounded by Sertoli cells and differentiating

Corresponding author:
Azar Sheikholeslami
Department of Mesenchymal Stem Cells, Academic Center for Education, Culture, and Research (ACECR), Qom Branch, Qom 3713189934, Iran
E-mail: azareslami@yahoo.com

spermatogonia [3, 4]. Although different kinds of testicular cells are involved in the creation of SSCs' niche, Sertoli cells are specialized components for SSCs regulation [5]. Sertoli cells associate closely with spermatogonia type A and secrete a variety of growth factors and cytokines that create an ideal microenvironment for SSCs [6]. In cancer patients, chemotherapy or radiotherapy may affect the process of self-renewal and differentiation of SSCs which may cause infertility by damaging the sperm production [7, 8]. Preservation of SSCs and saving them from damage in order to transplant in future, can be considered as a practical strategy to the treatment of germ cell-based male infertility [9-11]. So, different strategies such as using supportive cells or paracrine factors may be helpful in maintenance and differentiation of SSCs [12, 13]. The use of mesenchymal stem cells (MSCs) in regenerative medicine is gaining popularity since they face to no ethical problems of embryonic stem cells and can be collected from a variety of sources such as adipose tissue, bone marrow, and menstrual blood which all have a strong ability to develop into various cells and tissues [12]. MSCs have been demonstrated to release immunomodulating, anti-inflammatory, proliferative, and anti-apoptotic cytokines and growth factors, which are all packaged in extracellular vesicles (EVs). The adipose tissue-derived mesenchymal stem cells (AD-MSCs), as almost easy access and well characterized source of MSCs, have been widely employed for therapeutic purposes [12]. The most remarkable characteristics of AD-MSCs are high level of growth factors and cytokines secretion, high proliferation potential, and strong immunomodulatory effects [12]. Various studies have shown that conditioned medium (CM) derived from mesenchymal stem cells (MSCs-CM) contain the EVs which carry several proteins, such as chemokines, cytokines, and growth factors, lipids and nucleic acids which have potential to induce tissue regeneration or cell differentiation [13-16]. Regarding the beneficial effects of MSCs in regenerative medicine, and given that using CM could reduce some worries of using cells such as probability of tumorigenic effects, our experiment was designed. The aim of this study was to evaluate the proliferation and differentiation of rat SSCs in co-culture with Sertoli cells and using CM from AD-MSCs.

Materials and Methods

Experimental protocol

This experimental study was established on SSCs derived from testis tissue samples of neonatal rats purchased from Qom University of medical science. Moreover MSCs were isolated from adipose tissues which were obtained during liposuction surgery after obtaining written informed consent from volunteers. Ethical code was obtained from Research Ethics Committee of Mashhad Academic Center of Education, Culture and Research (IR.ACECR.JDM.REC.1397.012).

Isolation of SSCs and Sertoli cells from testicular tissue

First, testicular tissues were separated from 2–7 days old male rats and washed three times with phosphate-buffered saline (PBS). Then, mechanical digestion of the testicular tissue samples was done by insulin needles. After that, for enzymatic digestion, collagenase I was added for 15 minutes at 37°C. The resulting solution was centrifuged for 10 minutes at 1,800 rpm and the supernatant was removed. Then, it was exposed to trypsin-EDTA 0.25% for 15 minutes and centrifuged at 1,800 rpm for 10 minutes again. The supernatant was removed and the precipitate obtained from centrifugation was cultured in Dulbecco's modified eagle medium (DMEM)/low glucose medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco), supplemented with 40 ng/ml glial cell line-derived neurotrophic factor (GDNF, RC215-13; Biobasic) and 20 ng/ml basic fibroblast growth factor (bFGF, RC218-25; Biobasic). The ambient temperature for cultivating these cells was considered to be 34°C.

Characterization of SSCs and Sertoli cells by real-time polymerase chain reaction

In order to investigate the nature of SSCs and Sertoli cells, the expression level of some specific genes such as *c-Kit, integrin-β1, integrin-α6, Nanog* were evaluated by real-time polymerase chain reaction (PCR). Total RNA was extracted using the RNeasy kit (Gene All Biotechnology), and its amount was determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) at 260/280 nm. The reverse transcription was used to synthesize the first-strand cDNA using transcription Kit (Cinagen). The real-time PCR assay was carried out using RealQ Plus Master Mix Green (AMPLIQONIII), according to the manufacturer's instructions. The mixture comprised 10 μl of SYBR Green Master Mix, 1 μl of cDNA (equivalent to 1 ng of total RNA with an initial concentration of 500 ng/μl), 0.5 μl of PCR forward primer, and 0.5 μl of PCR reverse primer in a volume of 5 pmol/μl. Millipore water was also added to achieve a final

https://doi.org/10.5115/acb.22.246

volume of 20 μl. The primers for each gene were designed with OLIGO7 primer analysis software (Table 1). The experiments were carried out in triplicate for the quantification of all target genes. For normalizing gene expression levels, the GAPDH gene was used as an internal reference. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change of mRNA expressions for target genes. To characterization of SSCs and Sertoli cells by Immunocytochemistry to verify the co-culture of SSCs and Sertoli cells obtained from rats at the protein level, SSCs and Sertoli cells at passages 3–5 were cultured in 24-well plates. The cells were fixed with 4% paraformaldehyde (Sigma) for 10 minutes and washed with 0.4% Triton X-100 (Sigma) at room temperature. Cells were then blocked with blocking solution (10% goat serum; Sigma) for 30 minutes and incubated overnight at 4°C with anti-vimentin antibody (BioLegend) for Sertoli cells and anti-CD49f antibody (BioLegend) for SSCs. Then, for nucleus staining, they were exposed to 4',6-diamidino-2-phenylindole (DAPI) for 1–2 minutes, then washed three times with PBS/Tween-20 (PBST) and observed using an inverted fluorescence microscope (Olympus IX71).

Isolation and characterization of AD-MSCs

Adipose tissue samples were obtained from liposuction operation with written consent from patients and transferred from the hospital to the laboratory in physiological serum. The samples were washed several times with PBS and divided into smaller pieces. After mechanical digestion, collagenase I was added and incubated for 45 minutes at 37°C. Then, it was centrifuged for 10 minutes at 1,800 rpm and the supernatant was removed. The resulting cell sediment was cultivated in DMEM culture medium containing 10% FBS and 1% penicillin/streptomycin antibiotics and incubated in 37°C and 5% CO_2. To characterize the MSCs, their triple differentiation ability into 3 categories of bone, cartilage, and adipose was investigated under appropriate culture conditions. Then, real-time PCR was used to quantitatively measure the expression level of adipose-related gene (PPARγ), osteocyte-related gene (alkaline phosphatase), and chondrocyte-related gene (type II collagen). So, after trypsinizing the cells, total RNA was extracted (Gene All Biotechnology), the cDNA was made, and SYBR Green master mix was used to determine the expression value of the genes. The Sequences of primers are shown in Table 1.

Preparation of the CM

When the cells reached 70% confluence in the third passage, and 48 hours after culture media replacement, all the media was collected and passed through 0.22 filter. For next experiments, CM was saved in –70°C.

Treatment of SSCs with CM

In experimental group, when the culture dish of SSCs and Sertoli cells reached 70% confluence, the culture medium was replaced with the CM obtained from AD-MSCs and the cells were incubated in 34°C and 5% CO_2 for 12 days. In control group, SSCs and Sertoli cells received no treatment except DMEM with 10% FBS and 1X pen/strep. For better maintaining SSCs, GDNF (40 ng/ml) and FGF (20 ng/ml) were added to both experimental and control groups for whole 12 days. After 12 days, further analyses were performed to in-

Table 1. Sequence of real-time polymerase chain reaction primers

Gene name	Primer sequence	
	Forward primer (5'-3')	Reverse primer (5'-3')
ALPL	GAA GTC CGT GGG CAT CGT	CAG TGC GGT TCC AGA CAT AG
COL2A1	AAA GAC GGT GAG ACG GGA GC	GAC CAT CAG TAC CAG GAG TGC C
PPARγ	TGC CTA TGA GCA CTT CAC	TGA TCG CAC TTT GGT ATT
ITGA6	GCCAGTTGTGCTTGCTCTA	AGCGAGAAGCCGAAGAGG
ITGB1	TGCCAACCAAGTGACATAGAG	TCAATAGGGTAGTCTTCAGCC
c-Kit	GGCATCACCATCAAAAACG	GAGATGACTTGTTTCGGGC
Nanog	TCACACGGGCAAATACACG	CCAGGAAAAGTACGGCAGG
Stra8	CCTCTCTTCTTACTCTGCGA	ATCATCTCTGGGTTGGTTGC
Dazl	ACAACTTCTGAGGCTCCAAA	CTGGCAAAGAAACTCCTGAT
Scp3	TAGGCTTCGTCAGATGCTTC	CACCAGGCACCATCTTTAGA
Prm1	CAGCAAAAGCAGGAGCAGA	TAAAGGTGTATGAGCGGCG
GAPDH	AACCCATCACCATCTTCCAG	GTGAAGACGCCAGTAGACT

ALPL, alkaline phosphatase; COL2A1, type II collagen; ITGA6, integrin-α6; ITGB1, integrin-β1.

vestigate SSCs differentiation level.

Real-time RT-PCR for germ-like specific genes expression

After trypsinizing the cells, total RNA was extracted using RNeasy kit (Gene All Biotechnology) and the cDNA was made. The expression level of *Stra8, Dazl, Scp3,* and *Prm1* as germ-like specific genes was measured using RealQ Plus Master Mix Green as explained thoroughly before. The sequences of primers are shown in Table 1.

Immunocytochemistry for markers associated with male germ cells

After 12 days, the cells were fixed with 4% formalin for 10 minutes and permeabilized with 0.4% Triton X-100 for 10 minutes. Cells were then blocked with blocking solution for 30 minutes and incubated with anti-DAZL (orb156544, biorbyt), and anti-DDX4 (PA1963, bosterbio) overnight. The cells were washed with PBST and incubated with anti-Rabbit IgG FITC conjugated (AFIRAN, 1:500) dissolved in 5% BSA in dark (30 minutes at RT). The cells were washed with PBST and stained with DAPI for 1–2 minutes, followed by three times washing with PBST and observed using inverted fluorescence microscope (Olympus IX71).

Western blot for markers associated with male germ cells

After 12 days, the cells were trypsinized and lysed with lysis buffer. Total protein was extracted and the sample was run on polyacrylamide gel electrophoresis for 2–3 hours at 100 W. The obtained gel was used to transfer the protein to a poly vinylidene fluoride (PVDF) membrane. Then, membrane was incubated for 1–2 hours in room temperature with primary antibodies of anti-DAZL (orb156544, biorbyt) and anti-DDX4 (PA1963, bosterbio). The horseradish peroxidase (HRP)-conjugated Streptavidin-HRP conjugate (Abcam, 1:500) was added following the washing phase, incubated for 1–2 hours in diaminobenzidine (DAB) solution, and the DAB reagent was then removed. When the band was visible, the membrane was washed and analyzed.

Statistical analysis

Using *t*-test and U-Mann–Whitney, Fisher's chi-square, analysis of variance and follow-up tests, the results are analyzed.

Results

Characterization of SSCs and Sertoli cells

24 hours after isolation and cultivation, SSCs and Sertoli cells were attached to the bottom of the dish with numerous tiny colonies of almost round-shaped SSCs and fibroblast-like, spindle-shaped Sertoli cells which gradually increased during two weeks of culture (Fig. 1).

To better characterize the co-culture of SSCs and Sertoli cells, the expression level of some germ cell genes was investigated by real-time PCR. The results showed that *integrin-α6*, *c-Kit, integrin-β1*, and *Nanog* were clearly expressed in SSCs (Fig. 2).

Also, to confirm the co-culture of SSCs and Sertoli cells, we examined the expression of CD49f and vimentin markers for SSCs and Sertoli cells, respectively. The results of immunocytochemistry (ICC) showed that CD49f was expressed in SSCs, while vimentin expression was observed in Sertoli cells (Fig. 3A, B).

Characterization of the isolated AD-MSCs

To confirm multipotent adipose-derived stem cells, the triple differentiation ability of adipose-derived stem cells into 3 types of bone and cartilage fat was detected. The relative mRNA level of alkaline phosphatase was significantly higher ($P<0.05$; ANOVA) in AD-MSCs differentiated into the bone lineage than in control AD-MSCs (Fig. 4A). Also, relative

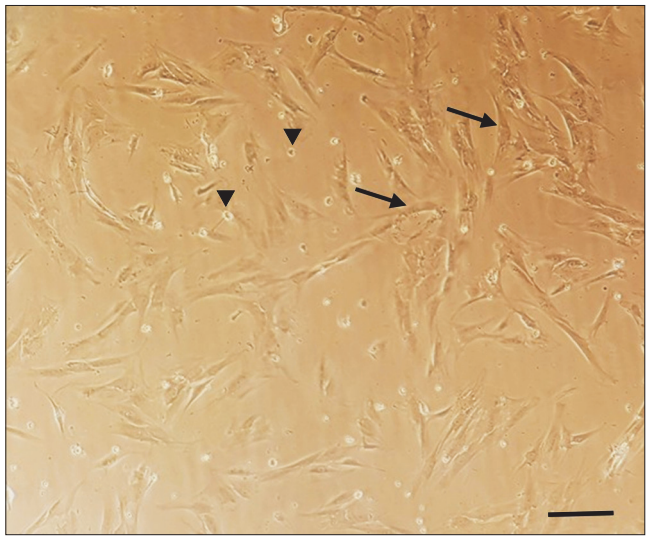

Fig. 1. Colonies of SSCs and Sertoli cells 72 hours after isolation. SSCs (arrowheads), Sertoli cells (arrows) (scale bar: 100 μm).

https://doi.org/10.5115/acb.22.246

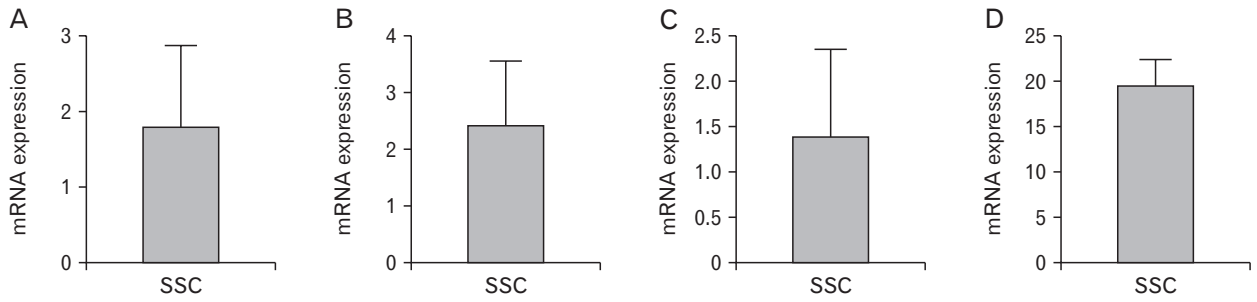

Fig. 2. The expression level of some germ cell genes. (A) *Integrin-α6* (*ITGA6*), (B) *c-Kit*, (C) *integrin-β1* (*ITGB1*), and (D) *Nanog* were clearly expressed in the co-culture of SSCs and Sertoli cells.

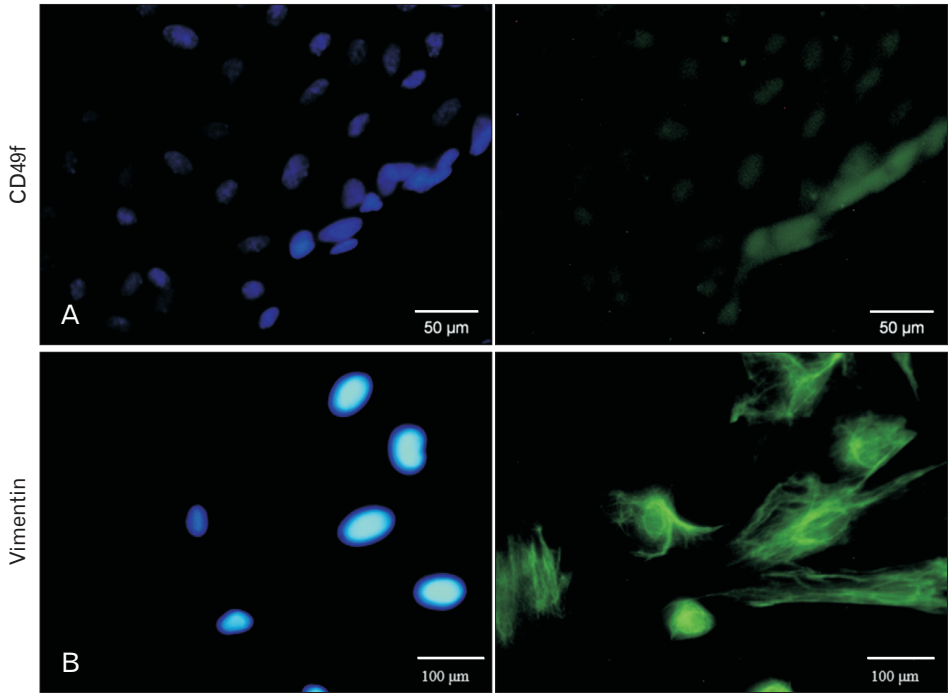

Fig. 3. Immunocytochemical staining to confirm spermatogonial stem cells (SSCs) and Sertoli cells. (A) CD49f (spermatogonial cell marker) was expressed in SSCs. (B) Isolated cells were also positive for vimentin (a marker of Sertoli cells). Nuclei were stained with DAPI (left figures).

expression of PPARγ and collagen type II was significantly higher (P<0.05; ANOVA) in AD-MSCs differentiated into adipocyte and chondrocyte phenotype, respectively, when compared with control AD-MSCs (Fig. 4B, C). In primary cultures, fibroblast-like and spindle-shaped AD-MSCs with distinct nuclei were detected by phase-contrast microscopy (Fig. 4D).

Evaluating germ cell-specific genes in SSCs after treatment

To determine the expression level of *Stra8, Dazl, Scp3,* and *Prm1* in SSCs, real-time PCR was used 12 days after treatment. There was no significant difference in the expression of pre-meiotic *Stra8* gene between CM-treated group and the control group (P=0.074). However, the expression level of *Dazl, Scp3*, and *Prm1* genes was significantly increased in treated group compared to the control group (P<0.0001, P=0.032, and P=0.003, respectively) (Fig. 5).

Immunostaining for evaluating the expression of germ cell markers

To investigate whether the treated group had experienced differentiation after 12 days, we examined the expression of DDX4 and DAZL as markers related to male germ cells at the protein level using ICC. The inverted fluorescent images showed that DAZL and DDX4 were expressed and detected clearly in treated groups rather than control group (Fig. 6).

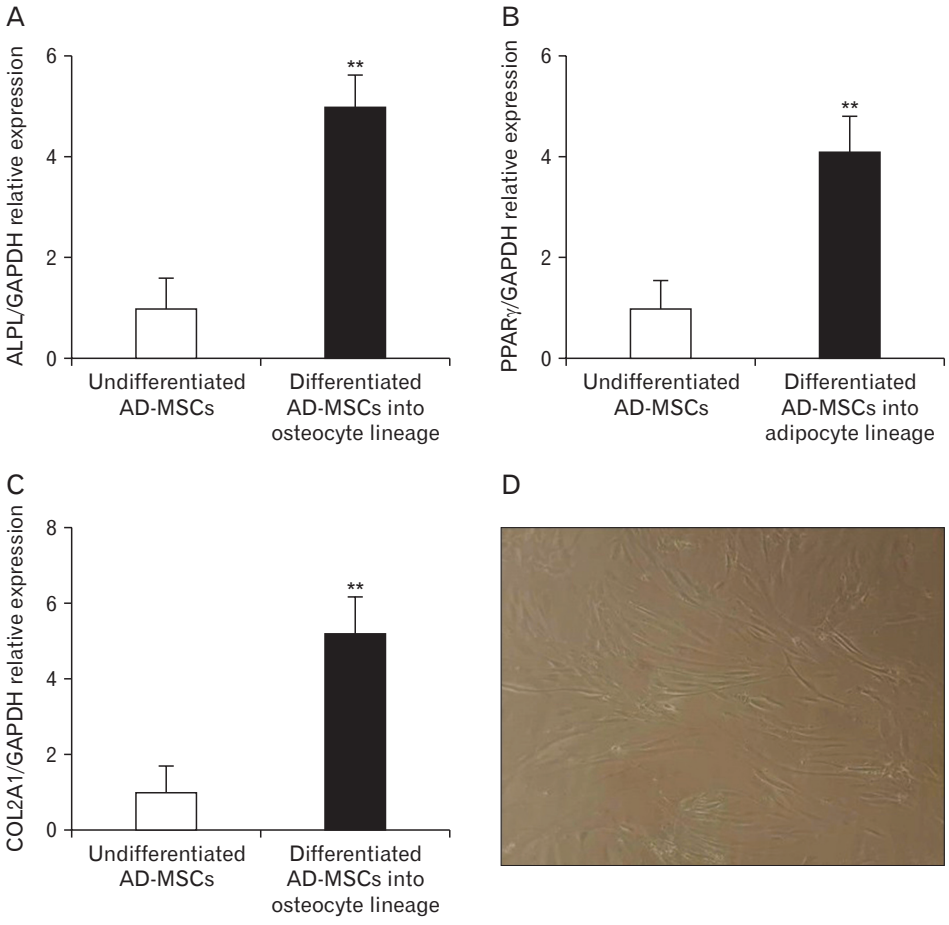

Fig. 4. The mRNA expression of (A) osteogenic, (B) adipogenic, and (C) chondrogenic markers was assessed after 14 days under specific media by real-time polymerase chain reaction in adipose tissue-derived mesenchymal stem cells (AD-MSCs) to confirm their capacity to differentiate into bone, fat and cartilage. (D) Inverted microscope image of living MSCs isolated from human adipose tissue showing spindle cells in the third passage. ALPL, alkaline phosphatase; COL2A1, type II collagen. ***P*≤0.01.

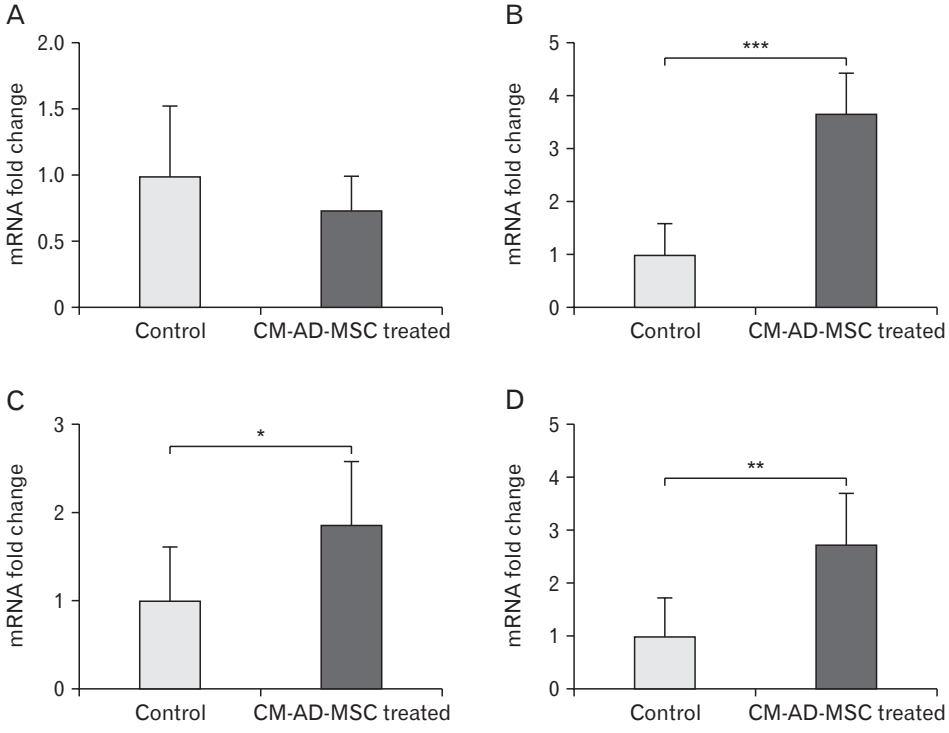

Fig. 5. Expression level of (A) *Stra8*, (B) *Dazl*, (C) *Scp3*, and (D) *Prm1* genes using real-time polymerase chain reaction in spermatogonial stem cells treated with CM after 12 days. CM, conditioned medium; AD-MSCs, adipose tissue-derived mesenchymal stem cells. **P*≤0.05, ***P*≤0.001, ****P*≤0.001.

https://doi.org/10.5115/acb.22.246

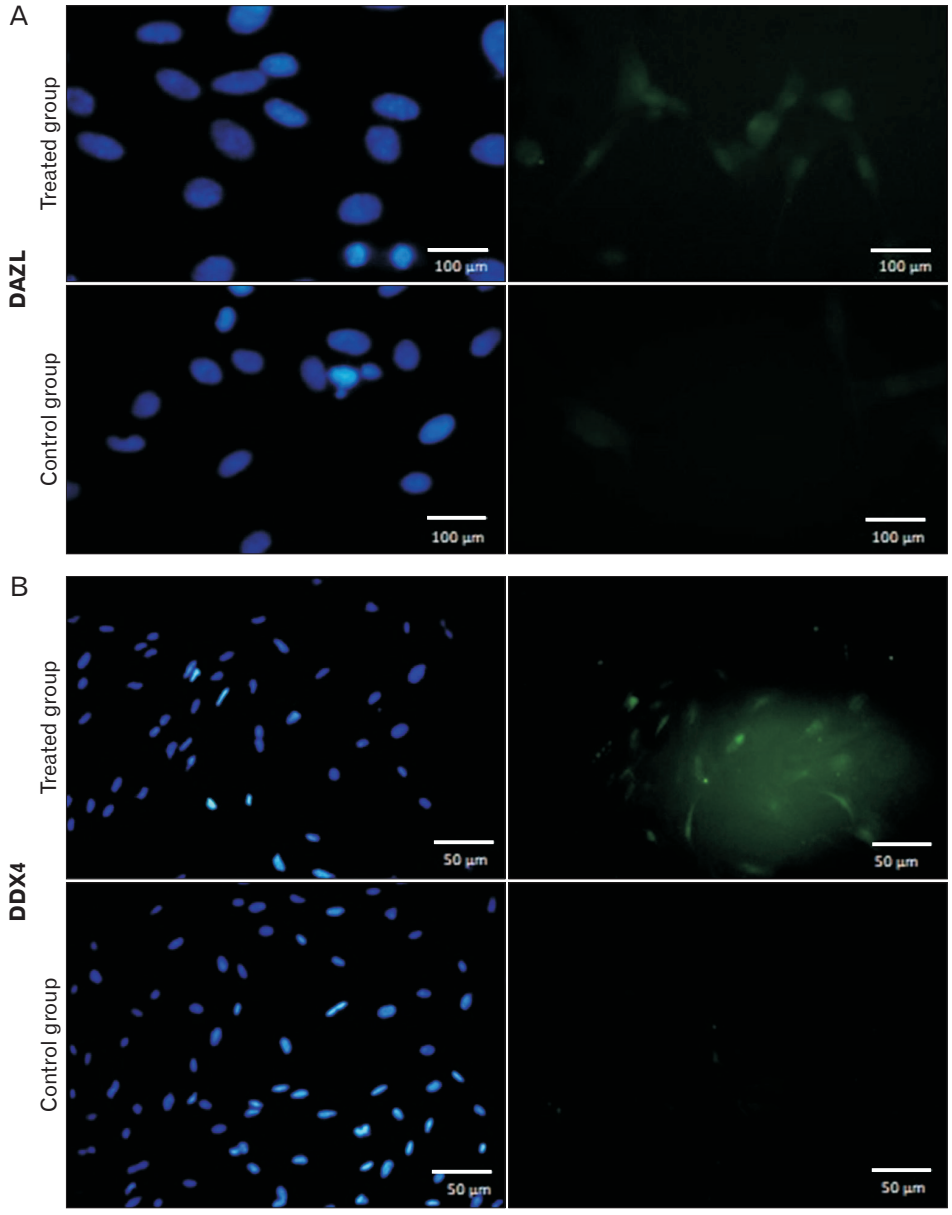

Fig. 6. Immunostaining of germ cell markers in the control and treat groups after 12 days. (A) Groups stained with anti-DAZL. (B) Groups stained with anti-DDX4. Right-hand images show nuclei stained with 4',6-diamidino-2-phenylindole.

Western blot for evaluating the expression of germ cell markers

The expression of male germ cell markers of DAZL and DDX4 was evaluated by western blot 12 days after treatment. The results of western blot indicated the presence of a strong single band (72 kDa) in all groups, which was equal to the target size of DDX4 protein (Fig. 7A). Also, a strong single band (35 kDa) was observed in all groups, which was equal to the target size of DAZL protein (Fig. 7A). DAZL protein showed no significant difference in the treated group than the control group (P=0.541) (Fig. 7B). Similarly, DDX4 protein had higher expression in treated group than the control group after 12 days treatment, however, the difference was not significant (P=0.291) (Fig. 7B).

Discussion

In recent years, a few therapeutic approaches have been proposed to conserve fertility in pre-pubertal boys, adolescents, and adult men, when chemo- and/or radiotherapy is required or in other causes-derived male infertility. For several years, the use of SSCs has been considered as a treatment option in all the mentioned cases [17]. These specific germ cells are able to generate a constant supply of differ-

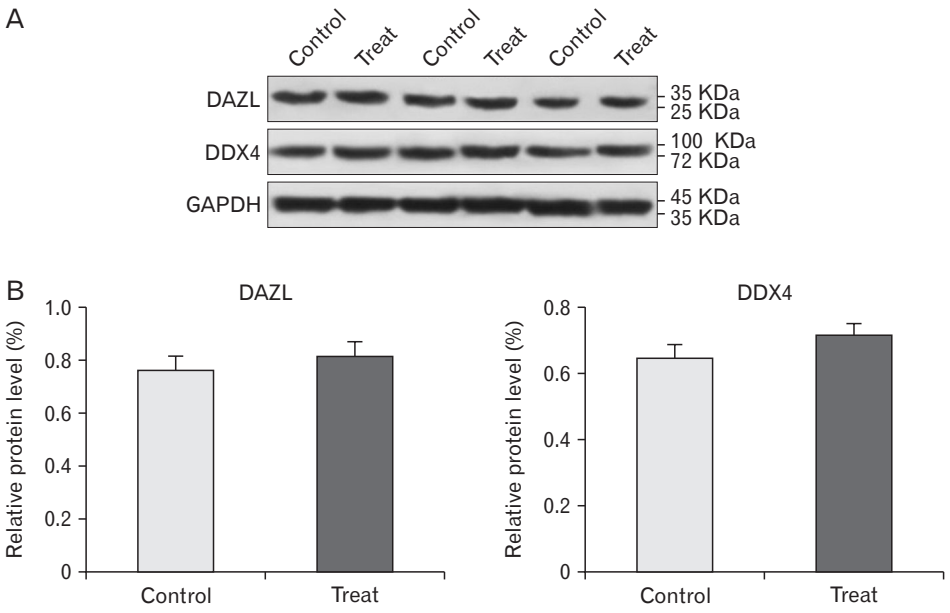

Fig. 7. Western blot for DAZL and DDX4 expression. (A) The expression pattern of DAZL, DDX4, and GAPDH on membrane. (B) Relative protein expression level of DAZL and DDX4.

entiated spermatogonia preserving spermatogenesis after the pre-pubertal onset of germ cell differentiation [18]. The signaling network of testis niche is responsible to provide a balance between self-renewal and differentiation of SSCs [19]. Beside, since it has been reported that anticancer therapies destroy this niche [9-11], transplantation of enriched SSCs as a practical way of preserving fertility will not be enough to restore fertility. So, co-transplantation of supportive cells or paracrine factors may be considered to optimize the transplantation procedure's efficiency. Although MSCs isolated from various sources, including adipose tissue, bone marrow, and umbilical cord, have been transplanted as an alternative for SSCs in a number of animal models, MSC-derived spermatogenesis has not been documented in any of these investigations [16, 20, 21]. Instead, the restoration of endogenous spermatogenesis was noted, suggesting the significance of the MSCs' secreted factors in the recovery of the testicular niche. Furthermore, in a recent study, the potential of MSCs in coordinating stem cell niche restoration through paracrine secretions was demonstrated [22]. In this study, the niche of SSCs as an open microenvironment where the processes of self-renewal and differentiation are in balance, was considered as an experimental model. The results showed that the subtonic injection of MSCs or their secretome lead to the recovery of spermatogenesis and the production of functional germ cells. The researchers concluded that MSCs could initiate tissue repair processes after injury by mimicking the function of supporting cells in the niche through paracrine activity [22]. It has been previously reported that co-transplantation of SSCs with MSCs in mice, leads to promoted fertility restoration efficiency [23]. However, since there is no definitive report on the long-term safety of MSCs yet, and due to concerns about potential tumorigenicity of MSC-based therapies [24], it seems that the application of MSC's derivatives, like their CM, can be considered as a proper alternative for cells themselves.

In this study, we found that the co-culture of SSCs with Sertoli cells in the presence of AD-MSCs-CM, GDNF, and bFGF resulted in more efficient differentiation, at least at the gene expression level. However, based on the findings of western blotting assays, greater expression of DAZL and DDX4 proteins in the treatment group compared to the control group was revealed, although it was not statistically significant (P=0.541 and P=0.291, respectively) (Fig. 7). *Dazl* is a master gene that controls the differentiation of germ cells, and Yu et al.'s [22] research suggests that its ectopic expression promotes the dynamic differentiation of mouse embryonic stem cells into gametes *in vitro*. Additionally, DAZL expression has been linked to the formation and differentiation of embryonic germ cells, and it is a feature of vertebrate germ cells, according to a study by Li et al. [23]. In details, we showed that the mRNA expression level of DAZL and *Scp3*, as meiotic genes, in the AD-MSCs-CM treated group was significantly increased in comparison to the control group (P<0.0001, P=0.032, respectively) (Fig. 5). Similar data was obtained in case of *Prm1* as a post-meiotic gene (P=0.003)

https://doi.org/10.5115/acb.22.246

(Fig. 5). However, comparing the expression level of pre-meiotic *Stra8* gene between AD-MSCs-CM treated and control groups showed no significant difference (P=0.074) (Fig. 5). Various studies have so far demonstrated that co-culturing Sertoli cells and SSCs in a media enriched with hormones promotes *in vitro* spermatogenesis [22-24]. In fact, it has been demonstrated that Sertoli cells and their endocrine and paracrine factors are crucial for maintaining SSC survival and differentiation *in vitro* [22]. In this regard, it was found that Sertoli cell transplantation can restore endogenous spermatogenesis in the irradiated rat testis whereas spermatogenesis was discovered in the tubules means that nearby the tubules that contained donor sertoli cells rather than in the donor cells containing-tubules which sertoli cells can induce differentiation of SSCs [22]. Beside, previous studies show that testis with MSC injections can recover spermatogenesis, but presumably not from transplanted cells. Nevertheless, more tubules containing spermatogenesis were found in the testes received MSC injections, suggesting a potential benefit of MSCs to boost the recovery of endogenous spermatogenesis following busulfan treatment [22, 23]. These findings imply that MSCs may aid in reestablishing endogenous spermatogenesis rather than differentiating towards the germline in order to improve restoring fertility. The paracrine substances released by MSCs may help SSC differentiation and restore the function of the niche. Vascular endothelial growth factor, hepatocyte growth factor, FGF2, angiopoietin (ANG) 1 and ANG2, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and many other paracrine factors that promote angiogenesis are secreted by MSCs [25, 26]. In addition, retinoic acid (RA) and TGF-β are considered as the key regulators in spermatogonial differentiation [27]. Our obtained results are in line with a study in 2012 on co-culturing Sertoli cells and SSCs, in which post-meiotic genes like *TP1*, *TP2*, and *Prm1* were up-regulated [28]. In that study, vitamins like vitamin C, E, and RA as well as hormones including testosterone and follicle-stimulating hormone were added to the culture system in order to mimic the natural niche as close as possible. However, in our study, we used CM of MSCs to provide a microenvironment similar to the natural niche of SSCs and support spermatogenesis.

Through architectural support and growth factor stimulation, the niche of SSCs provides the required stimuli to manage self-renewal and differentiation [29, 30]. GDNF and FGF2, involved in self-renewal, are some of these crucial factors [31]. Also, RA and TGF-β are considered as the key regulators in spermatogonial differentiation [27]. In light of this, it makes sense that the secretion of MSCs, such as CM or EVs like exosomes with the aforementioned secretory content, can improve the process and increase efficiency not only in the laboratory culture and differentiation of SSCs but also in designing clinical trials in order to their future transplantation.

ORCID

Hoda Fazaeli: https://orcid.org/0000-0003-4724-2106
Mohsen Sheykhhasan:
https://orcid.org/0000-0001-8599-7492
Naser Kalhor: https://orcid.org/0000-0001-9964-5607
Faezeh Davoodi Asl:
https://orcid.org/0000-0001-8346-8212
Mojdeh Hosseinpoor Kashani:
https://orcid.org/0000-0002-9547-0990
Azar Sheikholeslami:
https://orcid.org/0000-0001-8277-6539

Author Contributions

Conceptualization: AS, MS. Data acquisition: AS, MS, NK. Data analysis or interpretation: HF, FDA. Drafting of the manuscript: HF, MS, FDA. Critical revision of the manuscript: AS, HF. Approval of the final version of the manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Funding

None.

References

1. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. Nat Med 2008;14:1197-213.
2. Vander Borght M, Wyns C. Fertility and infertility: Definition and epidemiology. Clin Biochem 2018;62:2-10.
3. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, Vogelsong KM. World Health Organization reference values for hu-

man semen characteristics. Hum Reprod Update 2010;16:231-45.
4. Babakhanzadeh E, Nazari M, Ghasemifar S, Khodadadian A. Some of the factors involved in male infertility: a prospective review. Int J Gen Med 2020;13:29-41.
5. Kostereva N, Hofmann MC. Regulation of the spermatogonial stem cell niche. Reprod Domest Anim 2008;43(Suppl 2):386-92.
6. Vahdati A, Fathi A, Hajihoseini M, Aliborzi G, Hosseini E. The regenerative effect of bone marrow-derived stem cells in spermatogenesis of infertile hamster. World J Plast Surg 2017;6:18-25.
7. Goharbakhsh L, Mohazzab A, Salehkhou S, Heidari M, Zarnani AH, Parivar K, Akhondi MM. Isolation and culture of human spermatogonial stem cells derived from testis biopsy. Avicenna J Med Biotechnol 2013;5:54-61.
8. Meistrich ML. Effects of chemotherapy and radiotherapy on spermatogenesis in humans. Fertil Steril 2013;100:1180-6.
9. Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WH. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. Lancet Diabetes Endocrinol 2015;3:556-67.
10. Marettová E, Maretta M, Legáth J. Toxic effects of cadmium on testis of birds and mammals: a review. Anim Reprod Sci 2015;155:1-10.
11. Tröndle I, Westernströer B, Wistuba J, Terwort N, Schlatt S, Neuhaus N. Irradiation affects germ and somatic cells in prepubertal monkey testis xenografts. Mol Hum Reprod 2017;23:141-54.
12. Park KS, Bandeira E, Shelke GV, Lässer C, Lötvall J. Enhancement of therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. Stem Cell Res Ther 2019;10:288.
13. Almeria C, Weiss R, Roy M, Tripisciano C, Kasper C, Weber V, Egger D. Hypoxia conditioned mesenchymal stem cell-derived extracellular vesicles induce increased vascular tube formation *in vitro*. Front Bioeng Biotechnol 2019;7:292.
14. Bazoobandi S, Tanideh N, Rahmanifar F, Zare S, Koohi-Hosseinabadi O, Razeghian-Jahromi I, Dianatpour M, Ahmadi M, Khoradmehr A, Nabipour I, Khodabandeh Z, Tamadon A. Preventive effects of intrauterine injection of bone marrow-derived mesenchymal stromal cell-conditioned media on uterine fibrosis immediately after endometrial curettage in rabbit. Stem Cells Int 2020;2020:8849537.
15. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells *in vitro*. Biol Reprod 2003;68:2207-14.
16. Zhang D, Liu X, Peng J, He D, Lin T, Zhu J, Li X, Zhang Y, Wei G. Potential spermatogenesis recovery with bone marrow mesenchymal stem cells in an azoospermic rat model. Int J Mol Sci 2014;15:13151-65.
17. Vlajković S, Cukuranović R, Bjelaković MD, Stefanović V. Possible therapeutic use of spermatogonial stem cells in the treatment of male infertility: a brief overview. ScientificWorldJournal 2012;2012:374151.
18. Forbes CM, Flannigan R, Schlegel PN. Spermatogonial stem cell transplantation and male infertility: current status and future directions. Arab J Urol 2017;16:171-80.
19. Phillips BT, Gassei K, Orwig KE. Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 2010;365:1663-78.
20. Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R, Gromoll J, Engel W. Derivation of male germ cells from bone marrow stem cells. Lab Invest 2006;86:654-63.
21. Ghasemzadeh-Hasankolaei M, Eslaminejad MB, Sedighi-Gilani M. Derivation of male germ cells from ram bone marrow mesenchymal stem cells by three different methods and evaluation of their fate after transplantation into the testis. In Vitro Cell Dev Biol Anim 2016;52:49-61.
22. Yu Z, Ji P, Cao J, Zhu S, Li Y, Zheng L, Chen X, Feng L. Dazl promotes germ cell differentiation from embryonic stem cells. J Mol Cell Biol 2009;1:93-103. Erratum in: J Mol Cell Biol 2010;2:170.
23. Li H, Liang Z, Yang J, Wang D, Wang H, Zhu M, Geng B, Xu EY. DAZL is a master translational regulator of murine spermatogenesis. Natl Sci Rev 2019;6:455-68.
24. Barkholt L, Flory E, Jekerle V, Lucas-Samuel S, Ahnert P, Bisset L, Büscher D, Fibbe W, Foussat A, Kwa M, Lantz O, Mačiulaitis R, Palomäki T, Schneider CK, Sensebé L, Tachdjian G, Tarte K, Tosca L, Salmikangas P. Risk of tumorigenicity in mesenchymal stromal cell-based therapies--bridging scientific observations and regulatory viewpoints. Cytotherapy 2013;15:753-9.
25. Maumus M, Jorgensen C, Noël D. Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes. Biochimie 2013;95:2229-34.
26. Kadam P, Van Saen D, Goossens E. Can mesenchymal stem cells improve spermatogonial stem cell transplantation efficiency? Andrology 2017;5:2-9.
27. Moreno SG, Attali M, Allemand I, Messiaen S, Fouchet P, Coffigny H, Romeo PH, Habert R. TGFbeta signaling in male germ cells regulates gonocyte quiescence and fertility in mice. Dev Biol 2010;342:74-84.
28. Minaee Zanganeh B, Rastegar T, Habibi Roudkenar M, Ragerdi Kashani I, Amidi F, Abolhasani F, Barbarestani M. Co-culture of spermatogonial stem cells with sertoli cells in the presence of testosterone and FSH improved differentiation via up-regulation of post meiotic genes. Acta Med Iran 2013;51:1-11.
29. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001;414:98-104.
30. Scadden DT. The stem-cell niche as an entity of action. Nature 2006;441:1075-9.
31. Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A, Shinohara T. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Reports 2015;4:489-502.