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Original Article

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A Comparative Analysis of Culture Systems with Human Amniotic Mesenchymal Stem Cells

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Abstract

Background: A "indirect co-culture using mesh" system is commonly employed to maintain spermatogenesis in cancer patients undergoing chemotherapy and radiation. This study aimed to investigate the co-culturing of mouse spermatogonial stem cells (SSCs) with human amniotic mesenchymal stem/stromal cells (hAMSCs) in an optimized environment.

Methods: SSCs from 3-6-day-old mice (n=10) were indirectly co-cultured with hAMSCs via mesh for two weeks. Three groups evaluated: control, SSCs with conditioned media, and SSCs indirectly co-cultured with hAMSCs. Gene expression analyzed for Plzf, c-kit, Sycp3, and Prm1. Immunohistochemistry assessed Plzf, and flow cytometry evaluated c-kit and Plzf.

Results: Showed a twofold increase in Plzf-positive cells after 14 days of culture (76.47%, $p \leq 0.05$), with a significant elevation in Plzf gene expression observed in the conditioned media group (188.1±65%, $p \leq 0.05$). Conversely, the expression of the c-kit gene decreased significantly in both the conditioned media and "indirect co-culture using mesh" groups. Notably, Sycp3 and Prm1 expression levels significantly increased in the conditioned media group compared to the control. These findings suggest the potential of conditioned media as a novel feeder for promoting in vitro mouse spermatogenesis.

Conclusion: Our results demonstrate that the inclusion of growth factors, such as GDNF and BMP-4, along with conditioned media and an "indirect co-culture using mesh" system utilizing meshes with SSCs, significantly enhances SSC proliferation and differentiation. The optimized conditions media provided by hAMSCs offer a superior feeder compared to traditional "indirect co-culture using mesh" systems for promoting both the proliferation and differentiation of SSCs.

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Introduction

Infertility affects nearly 15% of the global population, and male factors contribute to roughly half of all cases (1, 2). Two of the most prevalent cancer therapies that result in infertility in people who recover are chemotherapy and radiation. Male fertility is maintained through sperm freezing or spermatogonial stem cell (SSC) preservation before chemotherapy. To preserve fertility in children with cancer, procedures such as testicular tissue (TT) graft, testicular tissue cryopreservation, and spermatogonial stem cell transplantation (SSCT) have been researched (3,

4). The SSCT approach involves cryopreserving testicular tissue before chemotherapy and then transplanting SSCs back into the testicles following cancer treatment (5). Small testicular tissue samples, insufficient SSCs to repopulate after transplantation, and the risk of cancer recurrence after transplanting primary tissue are all concerns regarding SSCs transplantation (5, 6). Therefore, various *in vitro* approaches for SSCs proliferation were examined (7). For example, the use of growth factors like GDNF (8), EGF, LIF, and FGF2 in a culture medium can result in massive clusters of proliferating SSCs (9) or the use of feeder cells (7, 10), conditioned media (11), and two-dimensional and three-dimensional culture systems (12) can have significant impacts on transplantation success (13). An indirect mesh-based co-culture system with supporting cells might be employed to maintain the pluripotency and proliferation of SSCs *in vitro*. An *in vitro* study in 2009 demonstrated that indirect mesh-based co-culture of adult mouse SSCs with Sertoli cells had a stronger impact on SSC colonization compared to growth factors and cytokines such as GM-CSF, SCF, and GDNF, with GDNF showing the greatest effect (14). In other studies, the influence of various types of Sertoli cells on the proliferation of mouse SSCs in a culture medium was investigated. Co-culture experiments were conducted using SSCs with embryonic Sertoli cells, adult Sertoli cells, and TM4 cells (a Sertoli cell line). Next, the expression of SSC-specific genes, including c-kit, Mvh, and ZBTB was assessed. It was revealed that co-culturing SSCs with embryonic Sertoli cells enhanced the proliferation rate of SSCs compared to adult Sertoli cells and TM4 (15). Human amniotic mesenchymal stromal cells (hAMSCs) may differentiate into numerous cell lines and express mesenchymal stem cell (MSC) markers such as Nanog and Oct-4 (16). GDNF, bFGF, LIF, and IGF-1 growth factors are secreted by these cells (17, 18), which are required for spermatogenesis (15). hAMSCs are a novel source of cells that could act as a feeder layer (19). They are expected to be effective in the presence of growth factors as a source with a high potential to promote SSC differentiation and proliferation.

Therefore, in this study, SSCs were isolated from neonatal mouse testicular tissue and subsequently identified. They were extracted from placental tissue and cultured *in vitro*. For the first time, SSCs were cultured with hAMSCs using two different systems, mesh-based co-culture and conditioned medium, for two weeks, after which their proliferation and differentiation were compared using real-time qRT-PCR (Plzf, c-kit, Sycp3, and Prm1), immunocytochemistry (Plzf and c-kit), and flow cytometry (Plzf).

Methods

In the first phase, SSCs were isolated from the testes of neonatal mice aged 3-6 days (n=10) and cultured under primary conditions for one week. In the second step, hAMSCs were isolated from amniotic membrane tissue and cryopreserved. Three experimental groups of spermatogonial stem cells were divided and cultured for two weeks. The study methodology is illustrated in the graphical abstract.

Preparation of amniotic membrane: Human amniotic membrane samples from healthy mothers without sexually transmitted diseases such as syphilis, hepatitis B, C, or AIDS were prepared and delivered to the laboratory in Hank's balanced salt solution (HBSS) following cesarean delivery. Each donor signed a consent form at Akbar-Abadi Hospital, Tehran, Iran (The ethics committee number: IR.IUMS.FMD.REC.1398.397).

Isolation of amniotic membrane cells: Collagenase I was used to isolate cells from the amniotic membrane. Amniotic fluid was collected from a 25-year-old mother who gave birth at Akbar Abadi Hospital (20). The placental sample was placed in a sterile Petri dish, transferred to a Class II laminar flow hood, and washed with sterile PBS after separating its amniotic and chorionic layers. To extract the MSCs from the amniotic membrane, the tissue was cut into small pieces, and incubated with collagenase in an incubator for one *hr*. To separate the epithelial cells, 1 *ml* of the trypsin-EDTA enzyme was added and incubated for three minutes. The enzyme impact was neutralized by adding DMEM-F12 medium with 10% FBS after the tissue pieces were removed.

The mixture was centrifuged at 1200 *g* for 5 *min* to collect the cells at the bottom of the tube. After discarding the supernatant, the cell pellet was re-suspended in fresh culture medium. Following a thorough cell count, each flask was shown to contain around 2×10^6 cells. The cells were transferred to a 25 cm^2 flask and incubated, with the culture medium replacement twice weekly. When the cell density inside the flask reached 80-90%, 0.25% Trypsin-EDTA was added, and the cells were detached from the flask bottom over 1 *min*. Cell suspention was centrifuged at 1200 *g* for 5 *min*.

Subsequently, the supernatant was discarded, and the cell pellet at the bottom of the round bottom tube was gently resuspended in fresh medium to obtain a homogeneous cell suspension. Finally, the tube's content was transferred into the 25 *ml* flasks (Figure 1A). The medium of the flasks was changed twice a week.

When the amniotic membrane MSCs reached about 70–80% confluence, the culture medium was removed, and the cells were washed twice with sterile PBS to eliminate serum residues. Serum-free DMEM/F12 medium was then added, and the flasks were incubated for 24 *hr* at 37 °*C* in 5% CO_2. After incubation, the medium was collected and centrifuged at 300×*g* for 10 *min* to remove floating cells, followed by 2,000×*g* for 20 *min* to remove cell debris. The supernatant was then filtered using a 0.22 *µm* syringe filter and stored at −80°*C* for future experiments. No serum-starvation protocol was applied during conditioned medium collection.

SSCs isolation: Three to six-day-old neonatal NMRI male mice (n=10) were provided by Razi Laboratory, Tehran, Iran. The animals were kept under standard conditions with a 12-*hr* light/dark cycle at the temperatures of 22-25°*C*. The animals were given free access to water and a sufficient amount of standard laboratory feed. Animals were treated in accordance with the guidelines of the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran (Approval ID: IR.IUMS.FMD.REC.1398.397).

After administering anesthesia, the testes of 10 mice were removed during each extraction. The testes were collected and washed in PBS supplemented with penicillin (100 *IU/ml*) and streptomycin (100 *µg/ml*). SSCs were isolated according to our previously described method (21). Briefly, the testes were incubated in Dulbecco's Modified Eagle Medium (DMEM/F12; Gibco Invitrogen, USA) containing 0.5 *mg/ml* collagenase IV (Sigma-Aldrich, USA) and 0.5 *mg/ml* trypsin (Sigma-Aldrich, USA) for 30 *min* at 37°*C* were eliminated. Through washing and centrifuging in DMEM/F12 medium the interstitial cells. A second similar digestion step was performed on plates containing the retrieved tissue/cells. SSCs and Sertoli cells were extracted by centrifugation after removing seminiferous cord fragments. SSCs were counted under a microscope and divided into 1 million cells per plate. Half of their cultural medium was replaced every two days.

Primary culture of SSCs: SSCs were cultured in DMEM/F12 supplemented with 10 *ng/ml* glial cell line-derived neurotrophic factor (GDNF; RP-1107, Royan Institute, Iran), 2% fetal bovine serum (FBS; Gibco Invitrogen, USA), 1% penicillin-streptomycin (15140-148; Gibco Invitrogen, USA), and 1% non-essential amino acids for 5 days at 37°*C* (11140-035; Gibco Invitrogen, USA). The cells were maintained in a humidified atmosphere with 5% CO_2, and the medium was replenished every other day. After 5 days, cell counts were determined using a hemocytometer, and 1×10^5 cells were seeded in each well of 6-well plates, with three replicates for each of the following experimental groups. In the mesh group, 1.5×10^5 amniotic MSCs were counted and seeded onto meshes, which were then placed in 6-well plates containing spermatogonial stem cells at the bottom, for 2 weeks co-cultured. In the media group, 1.5×10^5 amniotic MSCs were cultured in DMEM for 24 *hr*, the resulting conditioned medium, enriched with growth factors secreted by hAMSCs, filtered through a 0.22 *µm* filter, and mixed with an equal volume of fresh medium before being added to the SSCs. SSCs in the control group were cultured in DMEM medium alone, without the addition of a nutrient-supporting layer. All experimental groups were cultured with FBS for a period of two weeks, during which half of the culture medium was replaced every two days (Figure 1B).

Cell viability assessment

Trypan blue staining: Cell viability was assessed by Trypan blue staining both before and after primary SSC culture. In this assay, Trypan blue dye (Sigma-Aldrich, USA) penetrates dead cells and stains their nuclei, while viable cells with intact membranes exclude the dye. The total cell number was determined by summing the unstained (viable) and stained (non-viable) cells. After trypsinization and centrifugation, the cells' media were discarded, and 1 *ml* of serum supplemented culture medium was added to the resultant cell plate. 10 *µl* of the cell suspension was mixed with 2 *µl* of Trypan blue dye, and transferred to a hemocytometer slide. The total cell count and viability were evaluated under a light microscope.

MTT-based viability test: Mitochondrial activity of SSCs was assessed using the tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (21). MTT was added to the culture medium of each group and incubated

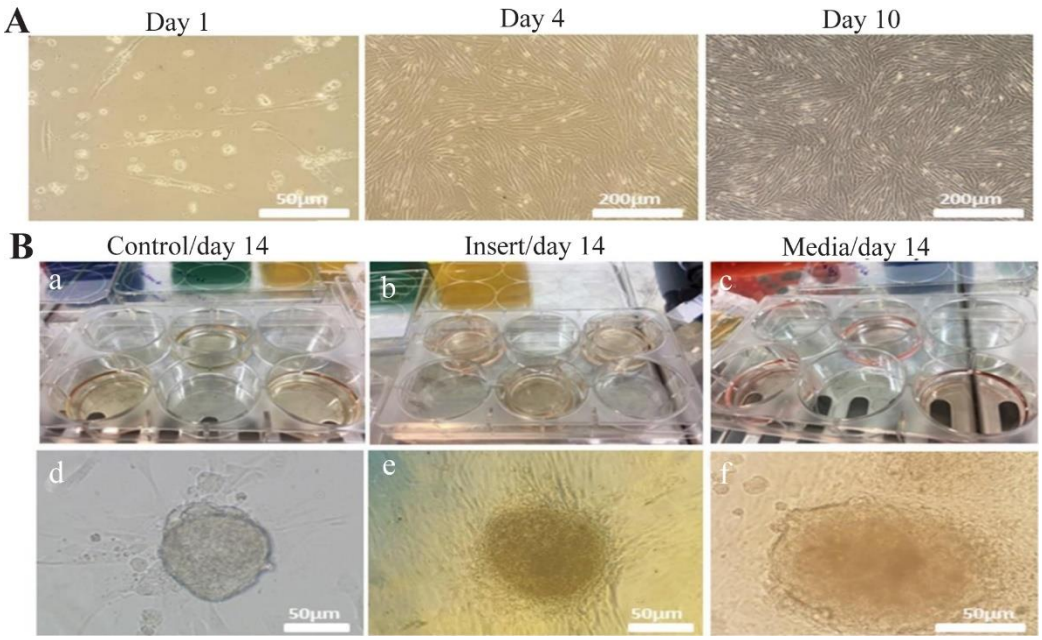

Figure 1. Characterization of SSCs in the experimental groups. A (a-c): SSCs were cultured in experimental systems, and the cells were evaluated on day 14. A (d-f): spermatogonial stem-like cell colonies were observed under inverted microscopy. In conditioned media and indirect mesh-based co-culture groups, most of the colonies began to disperse. B: Isolated from the amniotic membrane, MSCs were transferred to flask 25 and incubated for ten days. After 1, 4, and 10 days of cultivation, the images were captured. In all experimental groups, 1×10^5 SSCs were seeded in a six-well plate containing SSCs

for 4 *hr*. After 30 *min* of treatment with dimethyl sulfoxide (DMSO) and gentle shaking, the optical density (OD) of suspention was measured using a microplate reader at 570 *nm*, with a reference filter of 620 *nm*. All MTT assay results were obtained in triplicate and expressed as percentages.

RNA isolation and qRT-PCR: In all groups, SSCs were collected for gene analysis before and after primary culture and after 14 days of *in vitro* culture. A quantitative reverse transcriptase-polymerase chain reaction was used to evaluate gene expression. Plzf and c-kit (pre-meiotic), Sycp3 (meiotic), and Prm1 (post-meiotic) were evaluated as spermatogenesis-specific genes. For each replicate, 3×10^5 cells were harvested and kept in RNAprotect Tissue Reagent (Qiagen, Germany). To extract RNA from SSCs, the RNeasy Micro Kit (Qiagen, Germany) was used. The QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used to make cDNA according to the manufacturer's instructions.

Plzf, Sycp3, and Prm1 primers were designed using primer design software (AlleleID; Premier Biosoft International, USA) (Table 1). Quantitative real-time PCR was performed on an iCycler Thermal Cycler (Bio-Rad, USA) using SYBR Premix Ex Taq II (Tli RNase H Plus, RR820A; Takara Bio, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Applied Biosystems protocol as follows: initial denaturation at 95°*C* for 10 *min*, followed by 40 amplification cycles of 95°*C* for 15 *s* and 60°*C* for 1 *min*, and a final denaturation/melting sequence of 95°*C* for 15 *s*, 60°*C* for 15 *s*, and 95°*C* for 15 *s*. The final step was performed with a 2% ramp rate to generate a dissociation curve of the amplified products. Fluorescent reporter molecules were used to monitor amplification during each PCR cycle. β-actin served as the reference gene for normalizing mRNA expression levels, and the comparative CT method ($2^{\wedge-\Delta CT}$) was employed for analysis. All PCR reactions were conducted in triplicate.

Flow cytometry analysis: Flow cytometry was used to determine the percentage of undifferentiated SSCs expressing Plzf. After cell isolation, nearly 2×10^5 cells from all experimental groups were fixed in 4% paraformaldehyde (Merck, Germany) (pH=7.4) for 20 *min*. The cells were permeabilized for thirty minutes with 0.3% Triton X-100 (Merck, Germany) in PBS, and nonspecific connections were blocked for thirty *min* with 10% goat serum (Sigma-Aldrich, USA) in PBS. The samples were incubated overnight at

Table 1. The sequence of the designed primers used for qRT-PCR

Gene name	Gene bank code	*bp*	Primer sequences	Melt (°*C*)
c-kit	NM_021099.3	133	F: CCTCAAACAAGTCACCTCC	57
			R: GCTTTACCTGGGCTATGTG	58
Plzf	NM_001033324.3	103	F: ATTTACTGGCTCATTCAGCG	59
			R: CCAGTATGGGTCTGTCTGTG	60
Sycp3	NM_011517.2	82	F: TGGGATAGTTGAAGATGTTGGAG	57
			R: GGGAAGAAGAGGTTTGTTGATGT	58
Prml	NM_013637.5	180	F: AAGATGTAGTAGACGGAGGAGG	58
			R: ATTTTTCAAGATGTGGGGAGATG	57
β-actin	NM_009608.4	187	F: TCAGAGCAAGAGAGGCATCC	59
			R: GGTCATCTTCTCACGGTTGG	58

4°*C* with primary antibody solutions (1:100, anti-Plzf antibody: sc-28319; Santa Cruz Biotechnology, USA). After incubation and washing with PBS, the cells were exposed to the secondary antibody (goat anti-mouse IgG-FITC; Abcam, UK; AB 6785, AB6881, 1:200) for 60 minutes in the dark.

Flow cytometric analysis was performed using a BD FACSCalibur flow cytometer (BD Biosciences, Australia). Each experiment was conducted three times as independent biological replicates, using separate cell isolations for each experiment, and the results were analyzed using the WinMDI program, version 2.9 (The Scripps Research Institute, USA).

Immunohistochemistry: The slides were incubated with Plzf primary antibody and detected using an HRP-conjugated secondary antibody for immunohistochemical (IHC) labeling. All antibodies were human monoclonal IgG purchased from Santa Cruz Biotechnology, USA (Plzf, Cat. No. sc-28319). The staining was conducted according to the manufacturer's instructions (n=3 per condition from all treated placentas). Images of 10 randomly selected fields were captured using a light microscope. The images were analyzed using ImageJ software (version 1.8.0_112) with the Vessel Analysis plugin. The samples were fixed with 4% paraformaldehyde (Merck, Germany) and incubated for 20 *min* at 70°*C* in citrate buffer (pH=9.1). Subsequently the samples were treated with 0.2 *ml* of hydrochloric acid for 30 *min* for antigen retrieval and washed with PBS. Cells were permeabilized with 0.3%. Triton X-100 for 30 *min*. Next, 10% goat serum was added incubated as an additional background dye for 30 *min* to inhibit the secondary antibody response. The mixture sample was treated with primary antibodies (1:100; Sigma-Aldrich, USA).

Following the addition of secondary antibodies at a 1:150 dilution, the samples were incubated in the dark at 37°*C* for 1.5 *hr*. After four washes, the samples were removed from the incubator, kept in the dark, and treated briefly with DAPI, followed by a PBS wash. Finally, the samples were examined under a fluorescent microscope (Labomed TCS400) for immunocytochemistry analysis.

Immunocytochemistry: The cell suspension was cultured on sterile gelatin-coated slides. After 24 *hr*, the cells were rinsed with PBS and fixed in 4% paraformaldehyde for 4 *min* at room temperature. After washing with PBS, the samples were incubated in 2 N HCl for 20 *min* at room temperature. The samples were washed with PBS and then exposed to Triton X-100 for 30 *min*. It permits antibodies to enter the cell. Following that, the cells were incubated to 10% goat serum for 30 *min*. Goat serum proteins cover non-specific antigen sites to prevent non-specific responses. Primary antibodies were diluted in PBS (1:100; anti-Plzf, sc-28319, and anti-c-kit, sc-74569, both from Sigma-Aldrich, Germany) and incubated for 1 *hr*, followed by overnight storage at room temperature in a humid environment. Following two PBS washes, they were incubated with diluted conjugated secondary antibodies (1:200) for 60 *min* in the dark, then exposed to 37°*C*. After three additional PBS washes, the nuclei were labeled with DAPI and examined under a fluorescence microscope.

Statistical analysis: Statistical analyses were performed using Prism 7 and SPSS 16.0 software (SPSS Inc., USA). Data are presented as mean± standard deviation (SD) from three independent

biological replicates. Normality and homogeneity of variance were verified prior to analysis. Comparisons among experimental groups were conducted using one-way ANOVA followed by Tukey's post hoc test, with $p \leq 0.05$ considered statistically significant.

Results

Assessment of cell viability: After 14 days of incubation, spermatogonial stem-like cell colonies were clearly visible under and inverted microscopy. There were round colonies with clear edges and identifiable cells. Colony dispersion was observed, especially in the media and indirect mesh-based co-culture groups (Figure 1A). The viability of the SSCs from 3-6 days NMRI mice (n=10) after isolation and primary culture was 92.4±4% and 89.5±3%, respectively, as measured by Trypan blue staining. MTT-based test was utilized to assess viability throughout the culture phase (days 0 and 14). After 14 days of culture, the viability of cells in both the conditioned media and indirect mesh-based co-culture groups increased considerably relative to the control group (109.4±6.73%, 105.4±3.6%, 78.53±3%, mean±SD, respectively, $p \leq 0.05$). Additionally, cell viability in both experimental groups increased significantly on day 14 compared to day 0 ($p \leq 0.05$, table 2).

Immunohistochemistry staining: Plzf expression in mouse testes was shown to be spermatogonia-specific, as determined by immunohistochemistry. After two rounds of enzymatic isolation, Plzf-positive cells were found in 33.3% of neonatal mouse testis cells (Figure 2).

RNA isolation and qRT-PCR: In cells stimulated to proliferate by GDNF, c-kit and Plzf expression were assessed, while Prm1 and Sycp3 expression were evaluated to determine differentiation potential. On day 14, Plzf gene expression was significantly higher in the media group compared to the control group (188.1±65.14% and 42.33±28.13% respectively, $p \leq 0.05$). However, no significant increase was observed in indirect mesh-based co-culture group compared to the control (89.37± 49.28% and 42.33±28.13%, respectively, table 2, $p \geq 0.05$). The Plzf gene was upregulated in the media culture system compared to day 0 (188.1± 65.14% and 1.12±0.61%, respectively, $p \leq 0.05$).

Fourteen days after culture, c-kit gene expression was markedly downregulated in both the media and indirect mesh-based co-culture groups (0.08±0.05% and 0.25±0.24%, respectively, compared to 1.05±0.45% at day 0; table 3, $p \leq 0.05$). Sycp3 gene was upregulated in the control, media, and indirect mesh-based co-culture group after 14 days of culture (3.78±2.46%, 13.68±5.95% and 9.05±0.62%, respectively) with a significantly higher increase observed in the media group (from 1.02±0.26% to 13.68±5.95%, $p \leq 0.05$; table 3). A significant difference in the Sycp3 gene was observed between the control and media groups on day 14 (3.78±2.46%, 13.68±5.95%, respectively, $p \leq 0.05$).

Fourteen days after culture, Prm1 gene expression was upregulated in both the media and indirect mesh-based co-culture groups (media: 1.11±

Table 2. MTT test

Parameter	Day 0	Control/day 14	Media/day 14	Mesh/day 14	p-value
Cell viability (%)	100.0±0.000	78.53±3.001	109.4±6.734	105.4±3.600	$p \leq 0.05$

MTT assay results after 0 and 14 days of culture. Different superscript letters indicate significant differences between groups according to Tukey's post hoc test ($p \leq 0.05$). Data are presented as mean±SD (n=3)

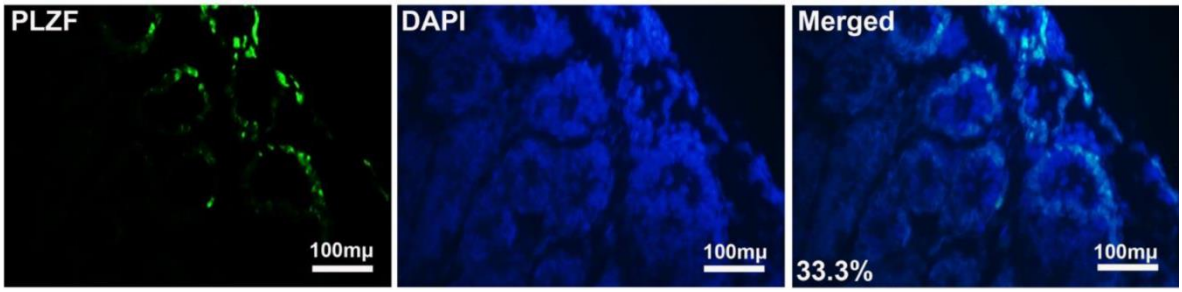

Figure 2. Immunohistochemistry staining. Plzf expression in mouse testes was evaluated by immunohistochemical labeling. All cell nuclei were stained with DAPI (blue), and Plzf-positive cells are shown in green. Scale bars: 100 μm

Table 3. Fold changes in gene expression levels measured by real-time PCR

Parameters	Day 0	Control/day 14	Media/day 14	Mesh/day 14	p-value
Plzf	0.0002215±0.00005714	0.001017±0.00057	0.002954±0.001286	0.001956±0.0001355	0.0124
C-kit	0.1026±0.04443	0.05822±0.02903	0.007917±0.004921	0.0252±0.02369	0.0181
Sycp3	0.0002215±0.00005714	0.001017±0.00057	0.002954±0.001286	0.001956±0.0001355	0.0124
Prm1	0.00003466±0.00001976	0.00006838±0.00001845	0.0006758±0.0002566	0.0004906±0.000206	0.0033

Quantitative gene expression analysis by qRT-PCR for premeiotic, meiotic, and post-meiotic SSC markers: (A) Plzf, (B) c-kit, (C) Sycp3, and (D) Prm1 after 14 days of culture. β-actin was used as the housekeeping gene to normalize expression levels. Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc test. Groups with different superscript letters are significantly different ($p<0.05$). Data are presented as mean±SD (n=3).

0.63% to 21.74±8.25%; indirect mesh: 15.78± 6.62% compared to day 0, $p\leq0.05$). Also, significantly higher Prm1 gene expression was observed in media and indirect mesh-based co-culture groups compared to the control group on day 14 (21.74±8.25%, 15.78±6.62%, and 2.2±0.59%, respectively, $p\leq0.05$, table 3).

Flow cytometric analysis of Plzf expression in mouse SSCs: The proportion of Plzf-positive cells, representing undifferentiated spermatogonial stem cells, was determined for each experimental group (Table 3). Following primary culture, 38.77± 4.2% of the isolated cells expressed Plzf. After 14 days of culture, a significant increase in Plzf-positive cells was observed across all groups, reflecting enhanced proliferation and preservation of stem cell characteristics in SSCs under the specified culture conditions. Specifically, Plzf-positive cells reached 61.13±2.98% in the control group, 76.47±2.55% in the conditioned media group, and 69.07±0.86% in mesh-based co-culture group, compared to 38.77±4.2% at day 0 ($p\leq0.05$, table 2). Among the groups, the conditioned media group showed the highest proportion of Plzf-expressing cells, approximately two-fold higher than the control, suggesting that soluble factors secreted by hAMSCs effectively promote SSC proliferation and preserve their undifferentiated state during *in vitro* culture.

Immunocytochemistry: Analysis of the data showed that Plzf expression increased significantly in the control, media, and mesh-based co-culture groups compared to day 0 (47.07±9.68%, 72±1.93%, and 65.8±2.98%, respectively, versus 16.93±4.7%; $p\leq0.05$; table 3). On day 14 of culture, Plzf expression was significantly higher in the media and mesh-based co-culture groups compared to the control group. Compared to day 0, expression of the c-kit differentiation marker decreased significantly in the control, media, and mesh-based co-culture groups (65.53±3.13%, 26.24±2.66%, and 36.27±3.41%, respectively, versus 80.6±1.68%; $p\leq0.05$; table 3). Additionally, on day 14 of culture, c-kit expression in the media group was significantly lower than in the control and mesh-based co-culture groups (Figures 3A and 3B).

Discussion

Many studies were conducted to find improved strategies for fertility preservation, particularly in individuals undergoing chemotherapy at younger ages (2, 22-24). The present study investigated the use of hAMSCs as a feeder layer and optimized culture conditions providing growth factors to promote SSC proliferation and differentiation. The A_{single} spermatogonial (A_s) cells are extremely rare cells that divide once every three days and make up about 0.03% of germ cells in the mouse testis (25). A_s, A_{pr}, and A_{al} constitute the population of undifferentiated spermatogonial stem cells that account for 0.3% of germ cells in the mouse testis (26). Various methods have been used to improve SSCs' culture conditions, such as adding different growth factors (17), using two-dimensional (27) or three-dimensional (12, 28) culture systems, or SSC transplants (29). Feeder layers have been used frequently for SSCs culture *in vitro* (10). Various feeder layers, such as Sertoli (14), umbilical cord (UC) (30), mouse embryonic fibroblast (MEF) cells, human embryonic stem cell-derived fibroblasts (hESCs) (31), SIM mouse embryo-derived thioguanine and ouabain resistant fibroblast cell line (32), and hAECs have been studied.

In fact, hASCs, which include hESCs and hAMSCs, have several advantages, such as regenerative capacity, multilineage differentiation potential, low ethical and legal concerns, and potent paracrine and immunomodulatory effects, which

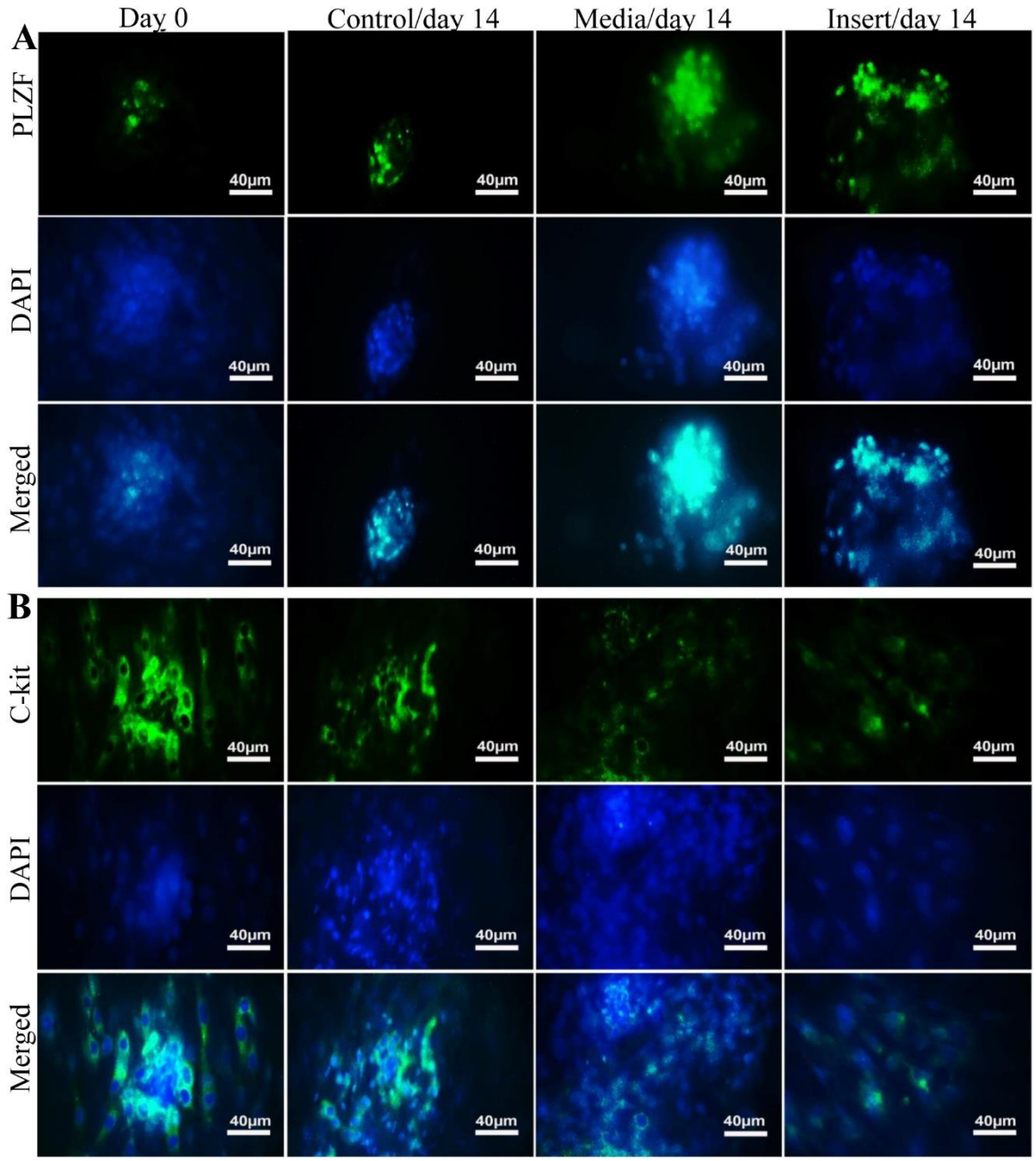

Figure 3. Immunocytochemical analysis of mouse SSCs. Colonies from all groups were examined at the start of culture (day 0) and after two weeks (day 14). Proliferative cells were identified using Plzf (green, A), differentiated cells using c-kit (green, B), and nuclei were counterstained with DAPI (blue). Scale bar = 40 μm

make them promising candidates for cell-based therapy. Transmission electron microscopy (TEM) analysis of hAMSCs revealed a more developed Golgi apparatus and rough endoplasmic reticulum (RER) compared with hCMSCs, consistent with enhanced secretory activity (16). Furthermore, this enhanced secretory activity of hAMSCs plays a key role by producing growth and differentiation factors such as FGF2, EGF, IGF, LIF, bFGF, and GDNF (33), which are critical for SSC proliferation and for preserving their core properties. Qiu et al. proved in their research that umbilical cord derived MSCs can differentiate into germ cells and overexpress their specific genes (34). Zhankina et al. were able to improve spermatogenesis in the testes of infertile mice using MSC-secreted exosomes, BMP4, and RA factors (35). Another study has shown that human

umbilical cord-derived MSCs can differentiate into a variety of functional cells and have a higher proliferation potential and lower immune resistance than human bone marrow mesenchymal stem/stromal cells (36). In 2015, Wang et al. recognized hAMSCs as a feeder layer for human bone marrow mesenchymal stem/stromal cells (hBMMSCs) to boost hBMMSCs proliferation and induce osteogenesis (37). Consequently, the present study was the first to compare hAMSCs in three different experimental groups with different conditions in the presence of essential growth factors for SSC proliferation and differentiation.

In our study, the SSCs were isolated from 3-6 days' mouse testes and underwent two stages of enzymatic digestion. SSC cells were primarily cultured for five days. Then, the SSCs were divided into experimental groups, and for the first time, the different culture systems were evaluated after two weeks. The experimental groups were as follows: (1) SSCs co-cultured with hAMSCs using a mesh system, (2) SSCs cultured with conditioned medium (collected from MSCs *in vitro*), and (3) a control group. The expression of proliferation-related genes (Plzf and c-kit) and differentiation-related genes (Sycp3 and Prm1) was measured by real-time PCR at the end of the primary culture (day 0) and after two weeks of *in vitro* culture (day 14). Flow cytometry was used to confirm the presence of proliferative SSCs in all groups at the start of culture and at the end of the second week. Also, immunohistochemistry was used to examine fresh testicular tissue samples using the Plzf and c-kit markers, and the results were compared to those on day 14. Colony formation of SSCs in the primary culture was evaluated by MTT assay over a 14-day period, from day 0 to day 14 of culture.

The viability of the SSCs from 3-6 days NMRI mice (n=10) after isolation and primary culture was 92.4±4% and 89.5±3%, respectively, as measured by Trypan blue staining. In 2021, Asgari et al. evaluated SSC viability using Trypan blue staining and reported 93±2%, which is consistent with our findings (12). Consistent with earlier findings, the MTT results after 14 days of culture showed a significant increase in cell viability in both the conditioned media and indirect mesh-based co-culture groups compared to the control group on days 14 and 0 (12).

In the current study, a substantial rise in the Plzf gene and protein expression was found in all groups after 14 days using three methods of qPCR, IHC, and flow cytometry, especially in the conditioned media group. Plzf protein levels increased significantly in all groups after 14 days, as confirmed by flow cytometry, qPCR, and immunohistochemistry. Plzf is a gene involved in the regulation of testicular cell proliferation (38). In mouse SSCs and their progenitor cells in the testis, Plzf is a nuclear-specific and proliferation-associated transcription factor (39, 40). Baazm et al. compared the effect of mature Sertoli, embryonic cells, and Sertoli cell line TM4 as a feeder layer on SSCs gene expression. The culture was carried out for two weeks, and the expression of c-kit and Plzf genes was analyzed using real-time PCR; the findings confirmed an increase in SSC-specific genes by the feeder layer (15).

The expression pattern of the c-kit gene reduced considerably after 14 days of culture in the media condition and indirect mesh-based co-culture groups compared to day 0. Our immunocytochemistry findings also supported this conclusion. The c-kit receptor, which is crucial for stem cell signaling, is predominantly found on the surface of hematopoietic stem cells. When stem cell factors attach to this receptor, differentiation and cell activity are triggered (41). The SCF/c-kit controls primordial germ cell motility, proliferation, and apoptosis throughout fetal gonadal development. The SCF/c-kit also affects SSC proliferation in adult animals. Moreover, c-kit also functions as a differentiation factor for rodent SSCs in the testes (42). In another study, the function of BMP4 in the 168-*hr*-long differentiation of mouse SSCs was investigated. The findings revealed that c-kit expression was higher in the early stages of development, but Plzf expression was lower in the later phases (43). Previous studies suggest that the two experimental groups, namely conditioned media and indirect mesh-based co-culture, are capable of modulating SSC proliferation and differentiation.

The expression level of the Sycp3 gene was considerably increased in the conditioned media group after 14 days of culture. Sycp3 shows germ cell differentiation, and its expression is low or nonexistent in A_s, A_{pr}, and A_{al} spermatogonia, but it continues to rise as spermatogenesis progresses (4). At each mitotic stage, Sycp3 is specific for spermatocytes with various nuclear patterns (44). The effect of Saikokaryukotsuboreito (SKRBT) on spermatogenesis and fertility in aging male mice resulted in enhanced sperm quality and Sycp3 expression in all groups (45). Ziloochi et al. (2020) cultured neonatal mouse SSCs on an

agar/polyvinyl alcohol (PVA) nanofiber scaffold, and 14 days after the differentiation phase, they observed the highest Sycp3 expression in the 3D group (46). Prm1 gene expression rose considerably in both the conditioned media and indirect mesh-based co-culture groups compared to the control group after 14 days of culture. Also, the Prm1 expression was significantly increased in the conditioned media group compared to the control group. The replacement of histones with protamines occurs continuously during spermatid maturation throughout spermatogenesis. Histones are replaced by protamine during the elongation phase, which involves a complex transfer of about 85% of histones. In nucleotide compression, the replacement of histones with protamine 1 and 2 is critical (47). Prm1 and Prm2 play an undeniable, vital, and functional role in organizing the nucleus of spermatid cells during the elongation phase of sperm development in mice. Heterozygosity in Prm1 or Prm2 reduces sperm nuclear density and can lead to male infertility (48, 49). A considerable increase in Prm1 and Acrv1 transcripts was observed after one month of 3D culture, indicating *in vitro* germ cell differentiation of SSCs using this technique (50).

Conclusion

The proliferation and differentiation of SSCs through two distinct co-culturing methods and conditioned media techniques utilizing hAMSCs modifications were investigated. Our results demonstrate that the inclusion of growth factors such as GDNF and BMP4, together with conditioned media and the indirect mesh-based co-culture system, significantly enhanced SSC proliferation and differentiation, as evidenced by our *in vitro* observations. Our findings suggest that the optimized media conditions provided by hAMSCs serve as a superior feeder compared to traditional mesh-based co-culture systems for promoting both SSC proliferation and differentiation. The results suggest that hAMSC-derived culture media could be commercialized, and with additional studies, they may become a valuable tool for preserving male fertility in clinical infertility settings.

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Conflict of Interest

The authors declare that there is no conflict of interest in this study.

References

1. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod. 2003;69(2):612-6.
2. Talebi A, Gilani MAS, Koruji M, Ai J, Rezaie MJ, Navid S, et al. Colonization of mouse spermatogonial cells in modified soft agar culture system utilizing nanofibrous scaffold: a new approach. Galen Med J. 2019;8:e1319.
3. Blatt J. Pregnancy outcome in long-term survivors of childhood cancer. Med Pediatr Oncol. 1999;33 (1):29-33.
4. Ghorbani S, Eyni H, Khosrowpour Z, Salari Asl L, Shabani R, Nazari H, et al. Spermatogenesis induction of spermatogonial stem cells using nanofibrous poly (l-lactic acid)/multi-walled carbon nanotube scaffolds and naringenin. Polym Adv Technol. 2019;30(12):3011-25.
5. Matulewicz RS, Meeks JJ. JAMA patients page. Testicular mass. JAMA. 2016;316(5):557.
6. Oatley MJ, Kaucher AV, Yang QE, Waqas MS, Oatley JM. Conditions for long-term culture of cattle undifferentiated spermatogonia. Biol Reprod. 2016;95(1):14.
7. De Rooij DG. The nature and dynamics of spermatogonial stem cells. Development. 2017;144(17): 3022-30.
8. Kanatsu-Shinohara M, Shinohara T. Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol. 2013;29:163-87.
9. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, et al. Generation of pluripotent stem cells from neonatal mouse testis. Cell. 2004;119(7):1001-12.
10. Hamidabadi HG, Bojnordi MN. Co-culture of mouse spermatogonial stem cells with sertoli cell as a feeder layer, stimulates the proliferation and spermatogonial stemness profile. Middle East Fertil Soc J. 2018;23(2):107-11.
11. Ghorbanlou M, Abdanipour A, Shirazi R, Malekmohammadi N, Shokri S, Nejatbakhsh R. Indirect co-culture of testicular cells with bone marrow mesenchymal stem cells leads to male germ cell-

specific gene expressions. Cell J. 2019;20(4):505-12.

12. Asgari F, Asgari HR, Najafi M, Eftekhari BS, Vardiani M, Gholipourmalekabadi M, et al. Optimization of decellularized human placental macroporous scaffolds for spermatogonial stem cells homing. J Mater Sci Mater Med. 2021;32(5):47.
13. Parolini O, Caruso M. Preclinical studies on placenta-derived cells and amniotic membrane: an update. Placenta. 2011; 32 Suppl 2:S186-95.
14. Koruji M, Movahedin M, Mowla SJ, Gourabi H, Arfaee A. Efficiency of adult mouse spermatogonial stem cell colony formation under several culture conditions. In Vitro Cell Dev Biol Anim. 2009;45(5-6):281-9.
15. Baazm M, Mashayekhi FJ, Babaie S, Bayat P, Beyer C, Zendedel A. Effects of different Sertoli cell types on the maintenance of adult spermatogonial stem cells in vitro. In Vitro Cell Dev Biol Anim. 2017;53 (8):752-8.
16. Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, et al. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop on placenta derived stem cells. Stem Cells. 2008;26(2):300-11.
17. Hofer HR, Tuan RS. Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther. 2016;7(1):131.
18. Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ. 2014;21(2):216-25.
19. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98(5): 1076-84.
20. Kouchakian MR, Koruji M, Najafi M, Moniri SF, Asgari A, Shariatpanahi M, et al. Human umbilical cord mesenchymal stem cells express cholinergic neuron markers during co-culture with amniotic membrane cells and retinoic acid induction. Med J Islam Repub Iran. 2021;35:129.
21. Shabani R, Ashtari K, Behnam B, Izadyar F, Asgari H, Asghari Jafarabadi M, et al. In vitro toxicity assay of cisplatin on mouse acute lymphoblastic leukaemia and spermatogonial stem cells. Andrologia. 2016;48(5):584-94.
22. Eslahi N, Hadjighassem MR, Joghataei MT, Mirzapour T, Bakhtiyari M, Shakeri M, et al. The effects of poly L-lactic acid nanofiber scaffold on mouse spermatogonial stem cell culture. Int J Nanomed. 2013;8:4563-76.
23. Navid S, Abbasi M, Hoshino Y. The effects of melatonin on colonization of neonate spermatogonial mouse stem cells in a three-dimensional soft agar culture system. Stem Cell Res Ther. 2017;8(1): 233.
24. Gholami K, Pourmand G, Koruji M, Sadighigilani M, Navid S, Izadyar F, et al. Efficiency of colony formation and differentiation of human spermatogenic cells in two different culture systems. Reprod Biol. 2018;18(4):397-403.
25. Nayernia K. Stem cells derived from testis show promise for treating a wide variety of medical conditions. Cell Res. 2007;17(11):895-7.
26. Fayomi AP, Orwig KE. Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. Stem Cell Res. 2018;29:207-14.
27. Tiwari JN, Tiwari RN, Kim KS. Zero-dimensional, one-dimensional, two-dimensional and three-dimensional nanostructured materials for advanced electrochemical energy devices. Prog Mater Sci. 2012; 57(4):724-803.
28. Ibtisham F, Honaramooz A. Spermatogonial stem cells for in vitro spermatogenesis and in vivo restoration of fertility. Cells. 2020;9(3):745.
29. Azizi H, Niazi Tabar A, Skutella T. Successful transplantation of spermatogonial stem cells into the seminiferous tubules of busulfan-treated mice. Reprod Health. 2021;18(1):189.
30. Maghen L, Shlush E, Gat I, Filice M, Barretto TA, Jarvi K, et al. Human umbilical perivascular cells (HUCPVCs): a novel source of mesenchymal stromal-like (MSC) cells to support the regeneration of the testicular niche. Reproduction. 2017;153(1): 85-95.
31. Chen B, Wang YB, Zhang ZL, Xia WL, Wang HX, Xiang ZQ, et al. Xeno-free culture of human spermatogonial stem cells supported by human embryonic stem cell-derived fibroblast-like cells. Asian J Androl. 2009;11(5):557-65.
32. Wei X, Jia Y, Xue Y, Geng L, Wang M, Li L, et al. GDNF-expressing STO feeder layer supports the long-term propagation of undifferentiated mouse spermatogonia with stem cell properties. Sci Rep. 2016;6:36779.
33. Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ, de Rooij DG. Propagation of bovine spermatogonial stem cells in vitro. Reproduction. 2008;136(5):543-57.
34. Qiu P, Bai Y, Pan S, Li W, Liu W, Hua J. Gender depended potentiality of differentiation of human umbilical cord mesenchymal stem cells into oocyte-Like cells in vitro. Cell Biochem Funct. 2013;31(5):365-73.
35. Zhankina R, Baghban N, Askarov M, Saipiyeva D, Ibragimov A, Kadirova B, et al. Mesenchymal

stromal/stem cells and their exosomes for restoration of spermatogenesis in non-obstructive azoospermia: a systemic review. Stem Cell Res Ther. 2021;12(1):229.

36. Xu Y, Hong Y, Xu M, Ma K, Fu X, Zhang M, et al. Role of keratinocyte growth factor in the differentiation of sweat gland-like cells from human umbilical cord-derived mesenchymal stem cells. Stem Cells Transl Med. 2016;5(1):106-16.
37. Wang Y, Yin Y, Jiang F, Chen N. Human amnion mesenchymal stem cells promote proliferation and osteogenic differentiation in human bone marrow mesenchymal stem cells. J Mol Histol. 2015;46(1): 13-20.
38. Dadoune JP. New insights into male gametogenesis: what about the spermatogonial stem cell niche? Folia Histochem Cytobiol. 2007;45(3):141-7.
39. He Z, Jiang J, Kokkinaki M, Dym M. Nodal signaling via an autocrine pathway promotes proliferation of mouse spermatogonial stem/ progenitor cells through Smad2/3 and Oct-4 activation. Stem Cell. 2009;27(10):2580-90.
40. Payne C, Braun RE. Histone lysine trimethylation exhibits a distinct perinuclear distribution in Plzf-expressing spermatogonia. Dev Biol. 2006;293(2): 461-72.
41. Blume-Jensen P, Claesson-Welsh L, Siegbahn A, Zsebo KM, Westermark B, Heldin C-H. Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. EMBO J. 1991;10(13):4121-8.
42. Mauduit C, Hamamah S, Benahmed M. Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update. 1999;5(5):535-45.
43. Li Y, Zhang Y, Zhang X, Sun J, Hao J. BMP4/Smad signaling pathway induces the differentiation of mouse spermatogonial stem cells via upregulation of Sohlh2. Anat Rec (Hoboken). 2014;297(4):749-57.
44. Ozaki Y, Saito K, Shinya M, Kawasaki T, Sakai N. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expr Patterns. 2011;11 (5-6):309-15.
45. Zang ZJ, Ji SY, Zhang YN, Gao Y, Zhang B. Effects of saikokaryukotsuboreito on spermatogenesis and fertility in aging male mice. Chin Med J (Engl). 2016;129(7):846-53.
46. Ziloochi Kashani M, Bagher Z, Asgari HR, Najafi M, Koruji M, Mehraein F. Differentiation of neonate mouse spermatogonial stem cells on three-dimensional agar/polyvinyl alcohol nanofiber scaffold. Syst Biol Reprod Med. 2020;66(3):202-15.
47. Akmal M, Widodo MA, Sumitro SB, Purnomo BB. The important role of protamine in spermatogenesis and quality of sperm: A mini review. Asian Pac J Reprod. 2016;5(5):357-60.
48. Haueter S, Kawasumi M, Asner I, Brykczynska U, Cinelli P, Moisyadi S, et al. Genetic vasectomy-Overexpression of Prm1-EGFP fusion protein in elongating spermatids causes dominant male sterility in mice. Genesis. 2010;48(3):151-60.
49. Gustafson EA, Seymour KA, Sigrist K, Rooij DG, Freiman RN. ZFP628 is a TAF4b-interacting transcription factor required for mouse spermio-genesis. Mol Cell Biol. 2020;40(7):e00228-19.
50. Topraggaleh TR, Valojerdi MR, Montazeri L, Baharvand H. A testis-derived macroporous 3D scaffold as a platform for the generation of mouse testicular organoids. Biomater Sci. 2019;7(4):1422-36.