The Epigenetic Assessment of Human Spermatogenic Cells Derived from Obstructive Azoospermic Patients in Different Culture Systems

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Purpose: Generating functional gametes for patients with male infertility is of great interest. We investigated different cultural systems for proliferation of SSCs derived from obstructive azoospermic patients.

Materials and Methods: Testicular cells were obtained from men with obstructive azoospermia. After enzymatic digestion process, cells were assigned to various groups: culture of SSCs in the dish without cover (control group), co-culture of SSCs with infertile Sertoli cells (I), co-culture of SSCs with fertile Sertoli cells (II), culture of SSCs on nanofiber (covered with laminin) (III), culture of testicular cell suspension (IV). Then cells were cultured and colony formation, gene-specific methylation (by MSP), quantitative genes expression of pluripotency (Nanog, C-Myc, Oct-4) and specific germ cell (Integrin α6, Integrin β1, PLZF) genes were evaluated in five different culture systems.

Results: Our findings indicate a significant increase in the number and diameter of colonies in IV group in compare to control group and other groups. Expression of germ specific genes in IV group were significantly increased ($P \leq 0.05$) and levels of expression of pluripotency genes were significantly decreased in this group ($P \leq 0.05$) compared with other groups. Gene-specific pattern of methylation of examined genes showed no changes in culture systems during the culture era.

Conclusion: A microenvironment capable of controlling the proliferation of cell colonies can be restored by testicular cell suspension.

Keywords: spermatogonial stem cells; proliferation; epigenetic; testicular cell suspension; obstructive azoospermia

INTRODUCTION

Fertility in men depends on the spermatogenesis process leading to the production of fertile male gametes. A specialized microenvironment (niche) affects the balance of self-renewal and spermatogonial stem cells (SSCs) differentiation[1,2]. Niches include various types of somatic cells, including Sertoli cells, myoid (peritubular) and Leydig cells and also extracellular matrix (ECM) that contribute to the development of spermatogenesis by producing various growth factors[3]. For example, Sertoli cells support the proliferation of SSCs by producing glial cell-derived neurotrophic factor (GDNF). In addition, Leydig and peritubular cells secrete colony-stimulating factor 1 (CSF1) that appears to be playing a role in the SSCs proliferation. ECM proteins, including laminin, have been reported to be involved with spermatogonia and therefore are considered an important component of the niche[4-6].

About 15% of couples are infertile, and male factor infertility affects around half of them. Infertility has made due to genetic variants and segregating alleles, epigenetic factors and environmental pollution and factors that are not known yet. It has been reported that azoospermia comprises 25% of male infertility cases[7].

In vitro spermatogenesis may be useful in the treatment of patients with non-obstructive azoospermia (NOA), e.g. patients with Sertoli cell abnormalities and spermatogenesis arrest. Assisted reproductive technologies (ART) generally treat obstructive fertility problems by eliminating pregnancy barriers, but in many NOA patients, male germ cells are either absent or underdeveloped or incomplete and therefore not suitable for ART. Transfer of cells from the in vivo damaged environment into a culture medium can help better maturation and production of viable sperm[8].

There are a limited number of SSCs in a testicle tissue removed from a testis. This makes it necessary to use in-vitro propagation of SSCs for repopulation of adult human testes. On the other hand, there are no valid surface-specific isolation markers for SSCs, therefore, in vitro characterization of spermatogenesis that allows

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Table 1. The demographic table related to the conditions for patients to enter the research.

Clinical and pathologic data of azoospermic men		Inclusion criteria	Exclusion criteria
FSH levels (mIU/ml)		15-1	≥31
Testicular volume (ml)		15-6	≤5
			≥16
Testicular histologic findings	Dynamic biopsy	Motile sperm(+)	Motile sperm(-)
	Pathology assessment	all Spermatogenic cells(+)	Maturation arrest or SCO

Sertoly cell only syndrome

in vitro propagation of human SSCs is the goal of researchers. Accordingly, there is a major need for culture systems that mimic the natural situation and provide functional testicular cells[9]. However, functional sperm production in humans has not yet been achieved in vitro[10].

Various techniques have been proposed for in vitro propagation of human SSCs, including different cultural systems, such as various feeder layers, improvement of niche, different culture substrates, addition of serum or using feeder- and serum-free medium, using different growth factors and supplements[11].

Three-dimensional (3D) culture has been introduced very recently and has been hypothesized to be able to mimic seminiferous epithelium developing male germ cells better. Previous studies have been able to obtain morphologically healthy sperm in a 3D matrix material, maturated by support from somatic testicular cells and the presence of gonadotrophins[12]. As suggested, the applicability of this culture technique requires further research[13,14].

While 3D culture has been shown to support spermatogenesis, it's optimal conditions remain unknown. During culture, the genetic and epigenetic stability of human SSCs should be considered. In this study, the effectiveness of various cultures in the propagation and enrichment of human SSCs derived from OA patients was investigated in order to provide an efficient system.

MATERIALS AND METHODS

Sample collection

Human testicular samples have been obtained from men with OA that were recourse to Shayan-Mehr Clinic (Tehran-Iran) for the treatment of infertility under the testicular sperm extraction (TESE) program and the remaining tissue was used. The use of human testicular biopsy samples and all steps of this research study were conducted with the authorization of the Tarbiat Modares University Research Ethics Committee (5212037-Tehran, Iran). Written consent was received from those who would like to participate in the study. The demographic table related to the conditions for patients to enter the research is given in **Table 1**.

Isolation and cultivation of human SSCs

One hour after biopsy, tissue samples were transferred to Dulbecco's Modified Eagle medium (DMEM; Gibco, Paisley, UK), supplemented with 14 mm $NaHCO_3$ (Sigma, St Louis, MO, USA), 100 IU/mL penicillin, single-strength non-essential amino acids and 100µg/mL streptomycin and then were brought to the laboratory. Samples were enzymatically subjected to two stages: the samples were incubated in a medium containing 0.5 mg/ml collagenase, 0.5 mg/ml trypsin, 0.5 mg/ml hyaluronidase, and 0.05 mg/ml DNase, For 30 minutes at 37 ° C. During this time, samples were pipetted several times by sampler. Every enzyme is bought from Sigma, USA. Tubes and spermatogenic cells were deposited using gradient gravity. The result of the first phase of enzyme digestion was the parts of seminal tubes entering the second stage of digestion for further digestion. For further removal of interstitial cells, the second digestion stage (45 min at 37°C) was performed in DMEM by adding fresh enzymes to seminiferous tubular fragments. The cells that are in the tubes, including spermatogonia and Sertoli, were released by centrifugation at 1500 rpm for 4 minutes at 37 ° C. Due to the small number of SSCs in initial biopsies to evaluate the different cultivation systems of SSCs, after enzymatic digestion process, the cells were cultured for 2 weeks in five cultural systems. In order to unify cultures, biopsy samples were weighed before enzymatic digestion and 2×105 cells were placed per 12-well culture plate.

After cell isolation, all specimens were incubated for two weeks in culture 34-StemPro with its complement (Invitrogen), 6 mg/ml –D (±) glucose, 100 µg/ml transferrin, 60 µM putrescine, 25 µg/ml human insulin, 30 µg/ml Pyruvic acid, 30 nM sodium selenite, 60 ng/ml Progesterone, 1 µ/ml –DL lactic acid, 5 mg/ml bovine serum albumin, 2 µM L-glutamine, 10 ng/ml Glial cell line-derived neurotrophic factor (GDNF), MEM sol-

Table 2. Sequence of primers designed for quantitative PCR analysis in culture evaluation.

Gene name	Primer sequence	Gene Bank Code	Band size (bp)
Integrin α6	FOR: 5′- AGT GTT TAT ACT ATG GAA GTG TGG-3′ REV: 5′- TAC TAT GCA TCA GAA GTA AGC CT-3′	NM_000210.1	106
Integrin β1	FOR: 5′- TCC AAA CTA CGG ACG TAA AGC-3′ REV: 5′- CCC TCA TAC TTC GGA TTG ACC-3′	NM_033668.1	75
PLZF	FOR: 5′- GTA CCT CTA CCT GTG CTA TGT G-3′ REV: 5′- TGT CAT AGT CCT TCC TTC ATC TC-3′	NM_001018011	80
C-Myc	FOR: 5′- TGT AAA CTG CCT CAA ATT GGA C-3′ REV: 5′- GGA TTG AAA TTC TGT GTA ACT GCT-3′	NM_001198530.1	177
Nanog	FOR: 5′- CCT TGG CTG CCG TCT CTG-3′ REV: 5′- GCA AAG CCT CCC AAT CCC-3′	NM_058176.2	131
Oct-4	FOR: 5′- TCT CGC CCC CTC CAG GT-3′ REV: 5′- GCC CCA CTC CAA CCT GG-3′	NM_001173531.1	202
TBP	FOR: 5′- CCA GCA TCA CTG TTT CTT GG-3′ REV: 5′- GGC TGT TGT TCT GAT CCA TG-3′	NM_003194	151

Table 3. Sequence of methylated and non-methylated primers specific for genes used in MSP.

Gene name	Primer sequence	Gene Bank Code	Band size (bp)
Integrin α6	TTGTAGTTTTCGGAATTAGGATTTC	100	Integrin α6 MF
	AATAATACCCTACACTAAACCGTCG		Integrin α6 MR
	TGTAGTTTTTGGAATTAGGATTTTG	101	Integrin α6 UF
	TAATAATACCCTACACTAAACCATCAC		Integrin α6 UR
Integrin β1	TTTTTTTTCGATTTTCGGTC	203	Integrin β1MF
	AAATACCGCGACCTTTAACG		Integrin β1MR
	TTTTTTTTGATTTTTGGTTGG	203	Integrin β1UF
	TAAAAATACCACAACCTTTAACACC		Integrin β1UR
PLZF	GAAGTCGTTTTTAAGTTTCGG	125	PLZF MF
	CTACAACGTCCAACCAAACG		PLZF MR
	TGAAGTTGTTTTTAAGTTTTGG	130	PLZF UF
	CTAACTACAACATCCAACCAAACAA		PLZF UR
C-Myc	GAAATTTTGTTTATAGTAGCGGGC	140	C-Myc MF
	AAAAAAACGAATCCTAACAACGAC		C-Myc MR
	AAATTTTGTTTATAGTAGTGGGTGG	140	C-Myc UF
	CAAAAAAACAAATCCTAACAACAAC		C-Myc UR
Oct-4	GAAAAAGGGAAAGTTTCGTTTTC	105	Oct-4 MF
	GTACCATACTCCAAACCAACGTA		Oct-4 MR
	AAGAAAAAGGGAAAGTTTTGTTTTT	109	Oct-4UF
	ACATACCATACTCCAAACCAACATA		Oct-4UR

uble vitamins, 10^{-4} Ascorbic acid, 10 µg/ml -d biotin, -25×10^{-5} Mercaptoethanol, 30 ng/ml beta-estradiol, 20 ng/ml human epidermal growth factor, 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml human leukemia inhibitory factor (LIF). In order to evaluate different culture systems in SSCs proliferation, after enzymatic digestion, these cells were cultured for two weeks in medium mentioned above in 5 different culture systems: culture of SSCs in the dish without cover (control group), co-culture of SSCs with infertile Sertoli cells (I), co-culture of SSCs with fertile Sertoli cells (II), culture of SSCs on nanofiber (covered with laminin) (III), culture of testicular cell suspension (IV).

Culture of SSCs in the culture dish without cover

Supernatants from the previous centrifugation were transferred to the dishes containing the culture medium and incubated for one night at 37 ° C. After incubation, Sertoli cells are adhering to the dish floor while others are in suspension. Then the cell suspension was collected and counted by a specific cell counting slide and about 3 wells per 12 well plates were cultured.

Cultivation of SSCs with infertile Sertoli cells

In order to prepare this group, the cell suspension derived from the second phase of the enzyme was divided and cultured in at 37°C for overnight. Then, the upper cell suspension of one part of the sample was trans-

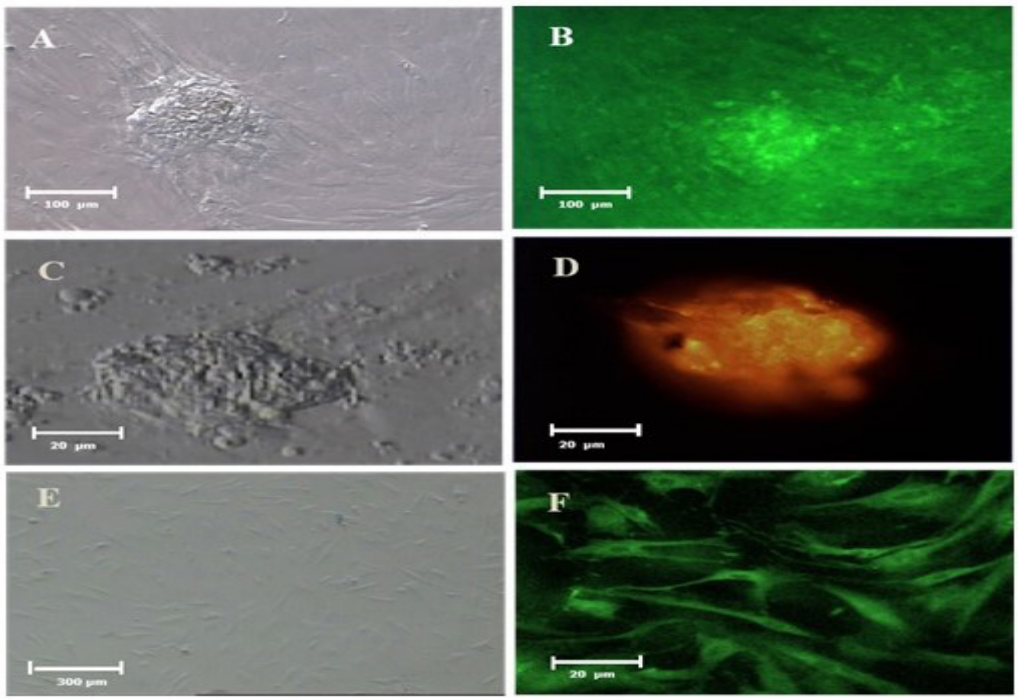

Figure 1. A and **C:** Human SSCs colony (A: Scale bars=100 µm and C: Scale bars=20 µm), **B:** Immunofluorecent staining of SSCs, detected Oct-4 positive under Immune fluorescence microscope (Scale bars =100 µm), **D:** Immunofluorecent staining of SSCs, detected PLZF positive under Immune fluorescence microscope (Scale bars=20 µm), **E:** Human Sertoli cells, (Scale bars=300 µm). and **F:** Immunofluorecent staining of Sertoli cells, detected vimentin, (Scale bars=20 µm).

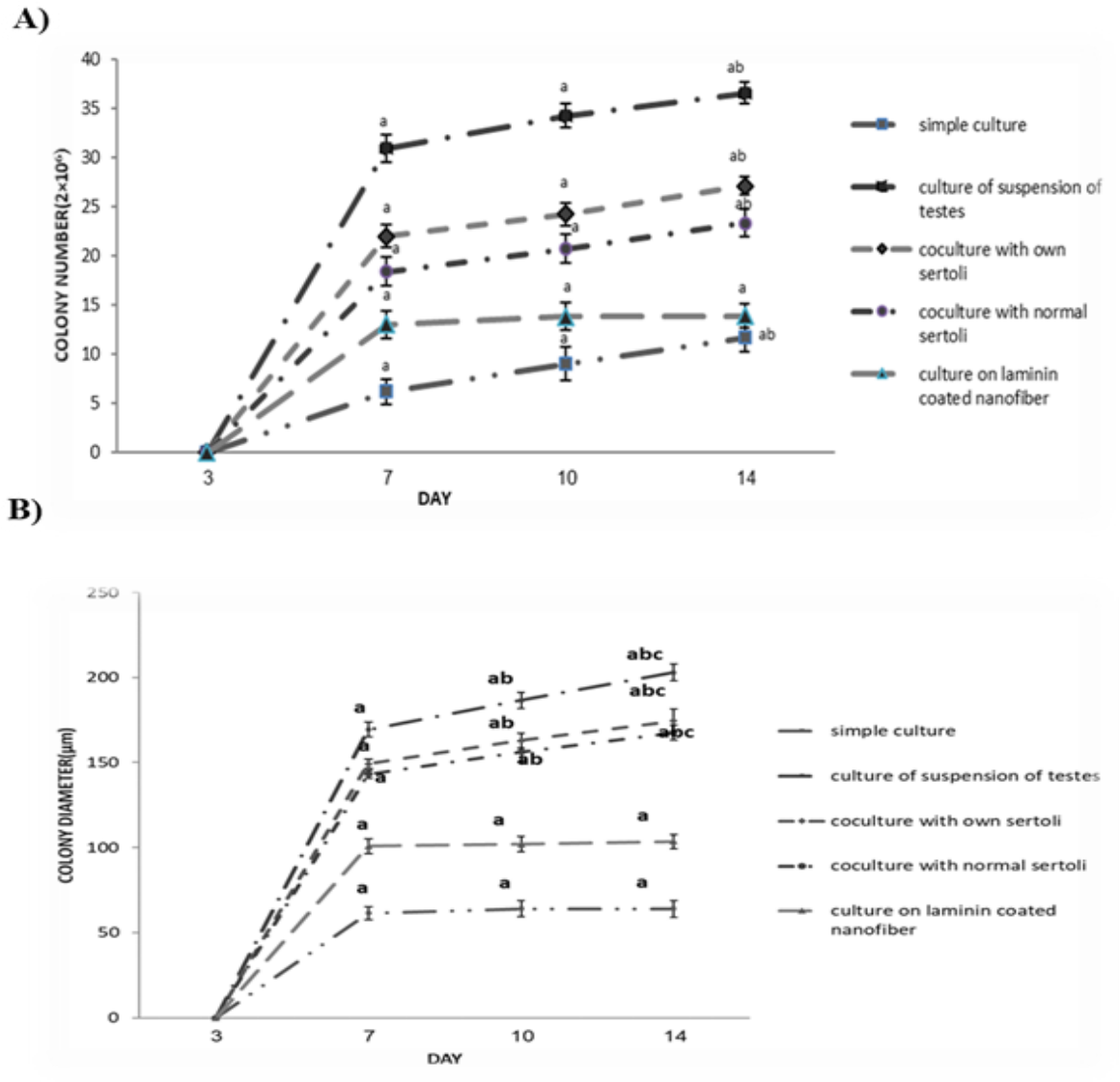

Figure 2. Comparison of colony number and diameter within cultural groups. a: Significant differences with day 3 in each group ($P \leq 0.05$). b: Significant differences with day 7 in each group ($P \leq 0.05$). c: Significant differences with day 10 in each group ($P \leq 0.05$).

ferred to another dish that after removal of the upper cell suspension, Sertoli cells were attached.

Cultivation of SSCs with fertile Sertoli cells

In order to isolate the Sertoli cells in this group, samples of the testicular biopsy were taken from fertile persons, who were undergoing orchidotomy according to the diagnosis of urologist. In order to prepare this group, the same method as the two groups mentioned, was used from cellular supernatants obtained from second-generation enzymatic digestion of healthy persons.

Culture of SSCs on nanofiber, covered with Laminin

Nanofibers were sterilized by immersion in 70% ethanol for 2 hours or sterilized by ultraviolet light, and then 20µg/mL laminin(Sigma-Aldrich) was added and covered nanofiber incubated for 2 hours until one night in 37 ° C. Before use, they were washed 1 to 2 times with phosphate-buffered saline (PBS) and they were ready for cell culture. In this group, cell suspension containing SSCs was used. The cells were load in such a way that in the beginning, the cells were mixed with 30 Landa of medium and about 3.5×105 cells/mL fiber were added slowly over fiber and then placed in incubator. After two hours, when cells were adhering to the fibers, the new medium was added to the laminin-coated dishes. The medium was replaced every other day.

Culture of testicular cell suspension

In order to prepare this group, the medium containing cells and seminiferous tubule components obtained from the first enzymatic digestion was centrifuged for 5 minutes at 1500 rpm (equivalent to 1500 RPM). The medium containing the enzyme was added to the cell sediment and the steps were continued in the order explained. In the end, after centrifugation, cell sediment was collected and all testicular tissue cells were cultured without isolation.

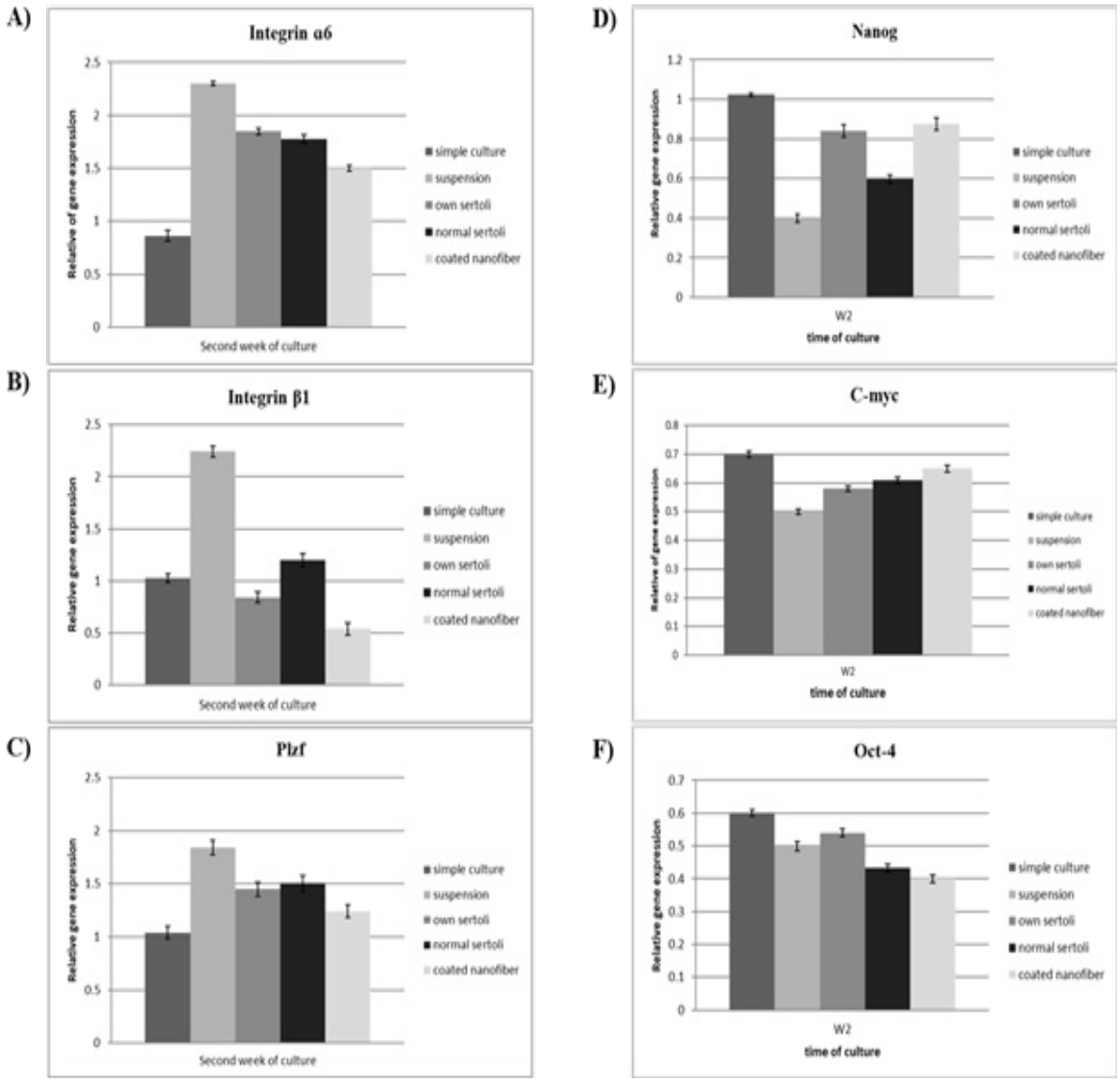

Figure 3. Gene expression pattern of Integrin α6 **(A)**, β1 **(B)**, PLZF **(C)**, Nanog **(D)**, C-Myc **(E)**, and Oct-4 **(F)** during SSCs culture in the studied groups. In each group, the expression level of gene in each sample is normalized to TBP, as an internal control. The level of expression of each sample is also calibrated to a calibratore (the cells derived from second enzymatic digestion). (N:3, Mean ± SD, $P \leq 0.05$)

Confirmation of Sertoli and SSCs

Immunocytochemistry was used for the identification of the Sertoli and SSCs. Initially, for fixing cells, 4% paraformaldehyde was used. After permeabilization with 0.2% Triton X100 and blocking with 10% goat serum (Vector, Burlingame, CA), the dishes received overnight incubation using mouse monoclonal anti-vimentin antibody (Sigma-Aldrich, USA) at room temperature (dilution ratio= 1:200). Then rabbit antihuman Oct-4 and Promyelocyte leukemia zinc-finger factor (PLZF) antibody (dilution ratio=1:100) was added at 4°C. After extensive washing with PBS, it was incubated at 4°C for 2 hours in the dark using the fluorescent-labeled secondary antibody (dilution ratio=1:100; Sigma-Aldrich, USA). The control dishes were under similar conditions but did not have the first antibody.

Evaluation of survival and cell proliferation

In order to determine the percentage of live cells, trypan blue and neobar slides were used and live and dead cells were counted in the leukocyte counter cells and the ratio and percentage were calculated. Also, to determine the total live cells in the sample, the live cells were counted in 1 mm3 sample.

Evaluation of Colony formation of SSCs

Colonies derived from SSCs, cultured over two weeks in all groups were studied on days 3, 7, 10 and 14 in terms of the number and diameter of the colony. Colonies' diameter was measured with an inverted microscope (Zeiss, Germany), equipped with graded eyepiece. Observations were repeated three times and mean and standard deviation were measured and analyzed.

Identity confirmation of SSCs

Quantitative analysis of gene expression:

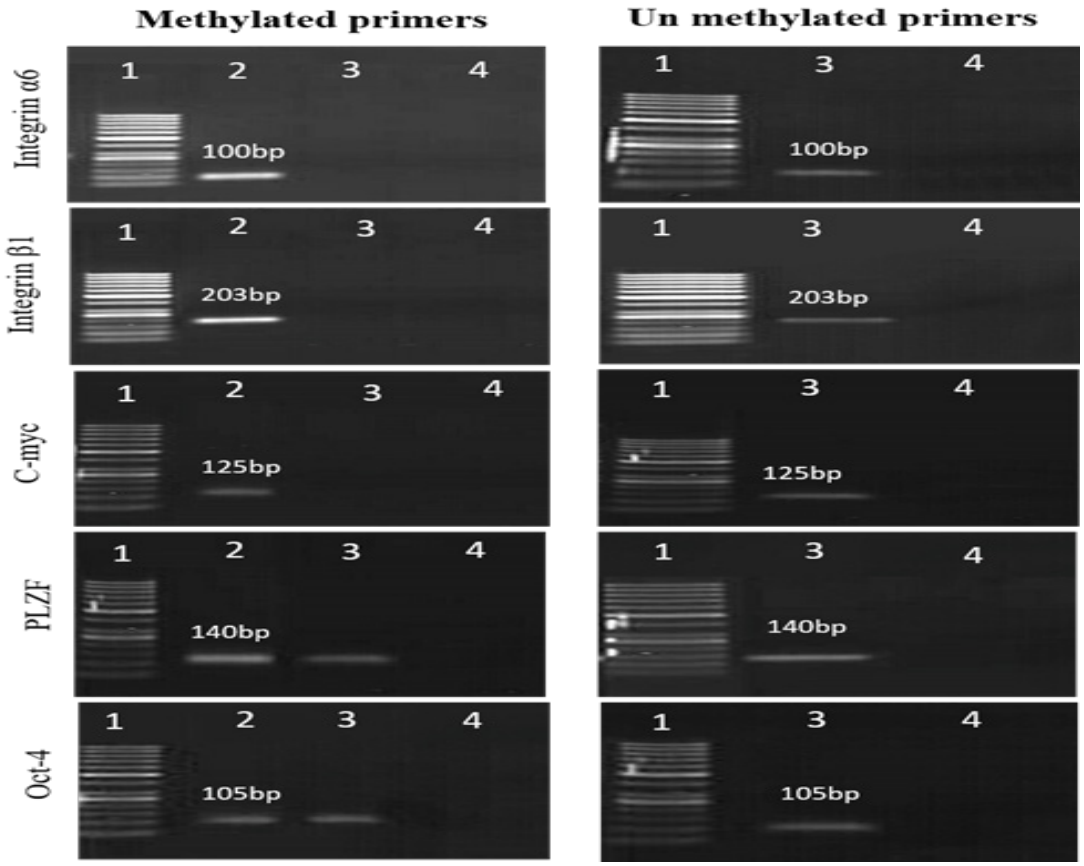

Figure 4. Results of MSP-PCR for methylation of genes: Integrin α6, β1, PLZF **(A)**, C-Myc, and Oct-4 **(B)**.

According to previous studies, assessment of spermatogonial genes expression was used to determine the presence of SSCs in culture. As a positive control, the total RNA was isolated from the testis, SSCs that were cultured on uncoated dishes, and SSCs derived from all cultures, using an RNX-Plus TM (Cinnagen, Iran) according to the recommendations of the manufacturer. Genomic DNA contamination was removed from extracted RNA, using DNase I (Fermentase) and RNA concentrations have been determined, using UV spectrophotometer (Eppendorf, Germany). Treated RNA was reverse-transcribed, using RevertAidTM first-strand cDNA synthesis kit (Fermentase) with oligo dT primer according to the recommended protocols. Oligonucleotide PCR primers specific for alpha-6-integrin, beta-1 integrin, PLZF, c-myc, Nanog, Oct-4 and TBP (internal control) genes were adapted from other primers and synthesized by GenFanAvaran Company. PCR master mix (Cinnagen) and SYBR Green were used for PCR reactions in a thermal cycler (Applied Biosystems, StepOne TM, USA). The cycling conditions included initial melting cycle (95°C for 5 sec) for polymerase activation, 40 cycles of melting (95°C for 30 sec), annealing (58-60°C for 30 sec), and annealing for an extended 30 seconds at 72 °C. Melt-curve analysis was used to confirm the quality of PCR reactions. To determine the efficiency, the standard curve for each gene was prepared, using serial dilution of cDNA from the testis. The reference and target genes for each sample were amplified simultaneously. The target genes have been normalized by the reference gene and expressed in relation to the calibrator. The comparative cycle threshold (CT) method was used to determine the gene expression ratio. Primers are listed in **Table 2**.

Epigenetic assessment

DNA extraction:

DNA of the SSCs in all groups were extracted using DNA extraction kit (Roche Co.) based on the suggested guideline at the end of the second week in a mechanical form, under reverse microscope guide. At the end of the second week, the cultured cells were isolated by trypsin and suspended after rinsing in 200 mL PBS. Then 200 mL binding buffer and 40 µL K proteinase were added and incubated in 70°C. Then, 100 µL isopropanol was added and centrifuged after being transformed into filtered tube. Finally, 50 µL elusion buffer was added and centrifuged. The quality of DNA extraction was tested on agarose gel by absorption in 260-280 nm wavelength.

Methylation of DNA by SSS1 enzyme:

To guide the methylate primers to DNA, SSS1 methylase enzyme (Biolabs Co, New England) was used according to the instructions and after treatment with sodium bisulfate (SBS), methylated primers were used for PCR. Enzyme stock 32 mmol was converted to 1600 mmol and incubated at 37°C for 1.5 hours. Heating up to 65°C for 20 minutes stops the reaction. Then methylated DNA was extracted using the kit and treated with SBS, before methylation-specific PCR (MSP) with M

primer. Finally, methylated DNA has been used as positive control in MSP with methylated primer (M).

Methylation-specific PCR (MSP):

MSP was performed with two primers: M primer with methylated DNA-modified sequence with SBS and, and U primer with non-methylated DNA-modified sequence with SBS. Proliferation with M primer showed methylation in CpG zones inside primer sequences and proliferation with U primer showed no methylation, while proliferation with both primers showed partial methylation in CpG zones inside primer sequences. In the present research, MSP with methylated and non-methylated primers was performed on Integrin α6, Integrin β1, PLZF, C-MYC, and Oct-4. Primers are listed in **Table 3**.

Statistical analysis

The quantitative variables were described as the mean and standard deviation (mean± SD) and categorical variable were expressed as the frequency and percentage. The results of cell proliferation by repeated measure analyses. To analyze the data of Real-Time PCR, first, the raw data were changed to reportable data through available formulas and then analyzed by One-way ANOVA. Statistical analyses were conducted in SPSS v.16 and graphs were prepared in Microsoft Excel 2007 software. The difference was considered statistically if $p \leq 0.05$.

RESULTS

The identification of colonies

Testicular biopsy specimens of OA patients after receiving were subjected to two stages of enzymatic digestion. In the first stage, the interstitial tissue was removed and in the second stage, the tubes were crushed and a suspension containing germ and Sertoli cells was obtained. Trypan blue staining of the isolated cells indicated viability of vast majority (> 90 %) of the cells. For the identification of colonies (Figure 1-A, C), we detected the expression levels of Oct-4 and PLZF by immunocytochemistry in different groups (Figure 1-B, D). The results showed that these markers were expressed in colonies after two weeks in proliferation media. Sertoli cells were used as a feeder for co-culture with SSCs (**Figure 1-E**), and were identified by vimentin (**Figure 1-F**). The results show the presence of this marker in the cytoplasm around the nucleus of Sertoli cells.

Assessment of colonization

The diameter and number of colonies in 5 culture systems were evaluated at days 7, 10 and 14. The colony count was significantly different between groups on different days; on day 7, mean colony count was higher in testicular suspension culture (30.9 ± 1.44), while it was lowest in simple culture group (6.2 ± 1.13) ($P < 0.05$). Mean colony count was 22 ± 1.15 in self-Sertoli culture, 18.4 ± 1.42 in healthy Sertoli cells, and 13.4±1.49 in culture on nanofiber covered with laminin. On day 10, mean colony count was higher in testicular suspension culture (34.2 ± 1.22) ($P < 0.05$), while it was lowest in simple culture group (9 ± 1.7). Mean colony count was 24.2±1.13 in self-Sertoli culture, 20.7 ± 1.49 in healthy Sertoli cells, and 13.8 ± 1.39 in culture on nanofiber covered with laminin. On day 14, mean colony count was higher in testicular suspension culture ($P < 0.05$), while it was lowest in simple culture group (11.7 ± 1.41). Mean colony count was 27.1 ± 0.87 in self-Sertoli culture, 23.3 ± 1.49 in healthy Sertoli cells, and 13.9 ± 1.19 in culture on nanofiber covered with laminin.

Also, there was an increasing trend in suspension culture and self-Sertoli cells in the consecutive days, which increased significantly on 7th, and 10th day than the third day ($P < 0.05$) and 14th day than 7th day, while it was not statistically significant on 14th day than 10th day ($P > 0.05$). In culture on nanofiber, there was a slight increase that was not statistically significant ($P > 0.05$). Mean colony count in simple culture group also increased from the 7th and 10th day than the 3rd day and 14th day than 7th day ($P < 0.05$); also, the increase on 10th than 7th day, and 14th than 10th day was not statistically significant ($P > 0.05$) (**Figure 2A**).

The colony diameter was significantly different between groups on different days; on day 7, mean colony diameter was significantly different among testicular suspension culture (169.4 ± 4.42), culture on nanofiber (100.9 ± 4.3), simple culture group (61.5 ± 3.86) ($P < 0.05$). Mean colony diameter was not significantly different among other groups; 149.2 ± 2.73 in self-Sertoli culture, 143.1 ± 2.5 in healthy Sertoli cells ($P > 0.05$). On day 10, mean colony diameter was significantly different among testicular suspension culture (186.6 ± 4.5), culture on nanofiber (102 ± 4.5), simple culture group (64 ± 4.7) ($P < 0.05$) and was highest in testicular suspension culture group ($P < 0.05$), while there was no statistically significant difference between self-Sertoli culture (163 ± 4.3), and healthy Sertoli cells (156.3 ± 6.11). On day 14, mean colony diameter was higher in testicular suspension culture (203.8 ± 7.4) than other groups ($P < 0.05$), while there was no statistically significant difference in colony diameter in self-Sertoli (174.5±6.9) than healthy Sertoli cells (167.6 ± 4.4) ($P > 0.05$) (Fig.2 B).

Also, there was an increasing trend in testicular suspension culture, simple culture, and culture on nanofiber in the consecutive days ($P > 0.05$), while means colony diameter significantly increased in self-Sertoli cells and healthy Sertoli cells on each day than the previous assessment day ($P < 0.05$).

Results of quantitative PCR for Integrin-α6, PLZF, Integrin β1, Nanog, C-Myc and Oct-4 gene

In the second week of culture, Integrin α6 expression and PLZF gene were significantly highest in testicular suspension cells culture (2.26 ± 0.05, 1.83 ± 0.02 respectively) and lowest in simple culture group (0.83 ± 0.04, 1.03 ± 0.002 respectively) than other groups ($P < 0.05$). In addition, although Integrin α6 expression was significantly higher in self-Sertoli culture (1.82 ± 0.06) than healthy Sertoli cells (1.73 ± 0.06), this difference was not statistically significant ($P > 0.05$) (**Figure 3-A, B**). Integrin β1 gene expression was expressed the highest in testicular suspension cells (2.25 ± 0.04) and lowest in culture on nanofiber (0.55 ± 0.06) group ($P < 0.05$), but was similar self-Sertoli (1.2 ± 0.01), healthy Sertoli (1.2 ± 0.07), and simple culture (1.28 ± 0.02) groups without significant difference ($P > 0.05$) (**Fig. 3-C**). Expression of C-Myc gene and Nanog were highest in simple culture (0.71 ± 0.03, 1.27 ± 0.03 respectively) and lowest in testicular cells group (0.50 ± 0.02, 0.38 ± 0.08 respectively) ($P < 0.05$), while it was not significantly different among self- Sertoli cells (0.59 ± 0.01, 0.83 ± 0.03) and healthy- Sertoli cells (0.60 ± 0.02, 0.59 ± 0.04), and nanofiber culture (0.64 ± 0.03,

0.87 ± 0.03) ($P > 0.05$) (**Fig. 3-D, E**). Oct-4 gene expression was highest in simple culture (0.65 ± 0.04) ($P < 0.05$). Although Oct-4 gene expression in nanofiber (0.40 ± 0.02) was lower than Sertoli cells groups (0.54 ± 0.04 in self- Sertoli and 0.44 ± 0.03 in healty- Sertoli), the difference was not statistically significant ($P > 0.05$) (**Figure 3-F**).

Results of MSP

The methylation pattern of Integrin α6, Integrin β1, PLZF, C-Myc, Oct-4 genes were evaluated during the SSCs culture using specific methylation PCR technique (MSP). Accordingly, MSP test was performed on the treated DNA of SSCs after the second week with methylated and nonmethyl primers. The results with methylated primer for Integrin α6, β1, and PLZF gene in cells in all studied culture systems follow a similar pattern and remained negative. However, MSP results for the non-methylated primer for these genes were positive in all groups. The size of the proliferation fragment for Integrin α6 for methylated primers was 100 bp and for non-methylated primers 101 bp, which were 203, and 205 bp, respectively, for Integrin β1 gene, and 125, and 130 bp, respectively, for PLZF gene. At the end of the second week, methylation pattern did not also change in C-Myc and Oct-4 gene during culture and it remained in partial Methylation. The size of the proliferation fragment was 140 bp for methylated and non-methylated primers in PLZF gene and 105 bp for Oct-4 (**Figure 4**).

DISCUSSION

In this research, we were able to show that the suspension of testicular cells could increase the forming of SSCs colony relative to other cultures. Spermatogenesis is a process that occurs in seminiferous tubules and controls several interactions between germ cell growth and the microenvironment surrounding it. Microenvironments are constituted by direct contact of the germ cells with somatic cells and acellular components that mediate proliferation and/or differentiation signaling to germ cells during spermatogenesis[3]. To rescue the fertility of patients with azoospermia, experimental approaches such as SSC transplantation, testicular tissue transplantation or in vitro germ cell maturation are under intense research development. Most researchers were therefore searching for structures of culture that can sustain this form of cellular interaction[15-18].

One of the problems in modeling the process of spermatogenesis is that until now, a suitable culture system has been not provided for the enrichment and proliferation of SSCs which could maintains nature and controlled ability of these cells. The co-culture of these cells or use of 3D scaffolds might be one way to achieve such goals[1,19] In this study, in order to establish an effective approach to human spermatogenesis in vitro, we investigated different culture systems to prolifrateand enrichment of SSCs derived from OA patients. In this study, five different systems of culture were used to proliferation of SSCs: simple culture system and culture of testicular cell suspension and co-culture system of SSCs with donor Sertoli cells, co-culture of SSCs with fertile Sertoli cells and culture of SSCs on Laminin-coated nanofibers. All systems relatively supported propagation and enrichment of SSCs, but the systems of testicular, Sertoli, and nano-fibers cells had more support, respectively.

It is noteworthy that in our study, the fate of SSCs was examined in a period of fourteen days and it is possible that different results will be obtained in longer cultures. Another point is that in this study, only six genes have been studied. Obviously, commenting on the efficiency of the selected cultural system to support cell proliferation or differentiation requires the study of a wider range of known genes and it is hoped that this will be considered in future research.

Our result showed significant differences in colony count among the groups with the highest values in testicular suspension, followed by self-Sertoli groups, and the lowest in the control group. Also, mean colony diameter was higher in testicular suspension group on day 7, and 10, compared to culture on nanofiber and simple culture but there was not significantly different among other groups; on day 14, In testicular suspension culture, the average colony diameter was higher than other groups ($P < 0.05$), while there was no significant statistical difference in colony diameter in self-Sertoli than healthy Sertoli cells ($P > 0.05$). Although some of the p-values were statistically insignificant, these results indicate higher colony diameter in all days in testicular suspension group than other groups.

Gene expression of germ cell genes showed significantly highest expression of Integrin α6 and PLZF in testicular suspension cells and lowest in simple culture group than other groups ($P < 0.05$), and Integrin β1 gene expression was highest in testicular suspension cells and lowest in nanofiber group, which confirmed that testicular suspension cells could effectively purify and enrich the functional human SSCs.

Gene expression of pluripotency genes showed the lowest expression of C-Myc gene and Nanog in testicular suspension cells, especially during second week in all groups ($P < 0.05$). Nevertheless, expression of Oct-4 gene was not significantly different among groups ($P > 0.05$). The results of gene expression also showed the superiority of testicular culture.

Germ-cell gene expression in testicular suspension cells increased with time, while pluripotency gene expression decreased by time. Different researchers have used various culture systems and have evaluated expression of various genes. Mirzapour and colleagues reported the largest number of colonies in the control group and the largest colony diameter in the presence of basic fibroblast growth factor (bFGF) and human leukemia inhibitory factor (LIF) on human Sertoli cells and positive SSCs markers, such as Oct4, Stra8, Piwil2, and Vasa, but negative for Nanog. They have suggested this technique efficient for human SSC colonization[20]. Other researchers have likewise suggested the favorable role of growth factors on human SSC culture[21], which could justify the results of the present study, indicating lower colonization in the control group (simple culture).

GFR-α1 (GDNF receptor) regulates SSCs niches and spermatogenesis maintenance through MAP kinase, NF-kB and IkB signal pathways[22]. The findings of the current study, in line with the above-mentioned studies, confirm that GDNF supplies the necessary items for growth and maintenance of human SSCs in medium.

Moreover, we found that pluripotency gene expression did not improve under the experimented culture conditions, while germ-cell gene expression increased. In addition, SSCs characteristics did not change and cells were not differentiated that shows the stability of this

culture technique, which could be due to the protective effect of GDNF against differentiation[23]. Besides the addition of other growth factors (e.g., EGF, FGF, LIF), feeder layer (such as Vero cells, mouse fetal fibroblasts (MEF), and Leydig and Sertoli cells) has also been identified as an important factor on human SSCs cultures[24].

According to the results of this study, the SSCs / Sertoli cells co-culture was better compared to the control group. For normal spermatogenesis, direct cell-cell interaction between the germ cell and feeder cell is important, and this relationship is not present in the monocultures of germ cell. Germ cell monocultures reduce survival and proliferation compared with co-culture of germ-feeder cells. Few studies have addressed the efficacy of co-culture with Sertoli cells.

Jabbari and colleagues used a soft agar system to amplify human SSCs by co-culturing with Sertoli cells. They concluded that the system reduces apoptosis and increases the proliferation of human SSCs[25]. Koruji et al. reported increasing the diameter and number of human SSC colonies by co-culturing SSCs with Sertoli cells[26]. In short-term co-culture with Sertoli cells, Tajik et al examined the effects of GnRH analog on SSC colonization[27]. Bahadorani et al demonstrated a short-term feeder-free culture of SSC for only one week in goat[28] which is consistent with the current study findings. Pramod and Abhijit[29] and others[20] the presence of stable colonies without any differentiation has been confirmed. This suggests that the long-term use of Sertoli cells as feeders may be appropriate for the spread of goat SSCs.

Nowroozi et al. obtained biopsy samples from 47 infertile patients who had NOA and grown in single-layer Sertoli cells. The size and number of colonies were assessed on days 8, 13 and 18. They observed that the process of differentiation and maturation of SSCs was stopped by increasing the colony size[30]. Other groups used 2D testicular mono- and co-cultures to show the required interaction between Sertoli and peritubular cells in the regulation of ECM expression as well as the testicular cell reorganization capabilities in vitro. Other groups used 2D testicular mono- and co-cultures to demonstrate the mutual interaction between Sertoli and the peritubular cells in regulating expression of ECM components and ability to reconstruct testicular cells in vitro[31,32].

By Inhibition caused by cell proliferation, the proliferation of Sertoli cells is reduced and FSH can restore this effect[33]. The superiority of co-culture with Sertoli cells in the present study is justifiable through GDNF-secreting Sertoli cells acting as SSCs renewal regulator. Because Sertoli cells derived from donor patient is homologous with human SSCs therefore it might be most suitable for culturing human SSCs and establishes a foundational feeder layer for the short-term goal of stable human SSCs cultures.

The use of 3D culture on nanofiber in the present study showed the superiority of this method than simple culture, regarding colony count and germ-cell specific gene expression. The three-dimensional culture, with the aid of the ECM, enables cells to organize properly and imitate spermatogonial epithelium. Various ECM has been used in three-dimensional cultures, like soft agar[34], and collagen gel matrix. Poly L-lactic acid (PLLA), used in the present study, has been previously used for other stem cells and has recently been used in human SSCs as one of the most promising biodegradable and biocompatible polymers approved by the US Food and Drug Administration agency which can form a three-dimensional non-woven network easily by electrospinning[1]. Eslahi et al. (2013) compared frozen-thawed SSCs seeded onto PLLA with the control groups (with no seeding on PLLA) and suggested the colony formation of human SSCs in the culture system can be increased by the PLLA, but may cause them to differentiate during cultivation[1] .

Sadri-Ardakani et al. succeed to reproduce a large number of human testicle spermatogonia cells in the presence of 34-StemPro medium. SSCs cells were able to hold germ cells for 28 weeks and 15 passages in the lab using laminin-plated dishes. It was observed that is induced the process of differentiation by placing SSCs on laminin[35,36]. Lim et al. documented the proliferation of isolated SSCs from obstructive and non-obstructive patients in the presence of growth factors such as GDNF, LIF, EGF and FGF on laminin-coated plates [37]. Koruji et al. used laminin-ECM-coated dishes with GDNF, EGF, LIF, and bFGF supplements to improve the human culture SSCs culture results[36]. In this study, which is consistent with the findings of the above-mentioned studies, we successfully used 3D nanofiber culture with laminin surface.

Some procedures, including cellular isolation and cultivation, can affect the SSC's integrity. The genome, epigenum, or both can alter in manipulated cells. Reports show that genetic stability exists in other populations of stem cells during in vitro culture. Since SSC is the cells that convey genetic information to the next generation, SSC's genetic stability is more important than somatic cells. Shinohara and colleagues showed that in vitro culture of mouse spermatogonia for more than 24 months was associated with karyotype and imprinting stability. The recipient mice's offspring were fertile and also had a typical imprinting pattern. However, the genetic and epigenetic changes of human SSC cultivated have not yet been determined. in agreement with other culture reports[21,38], and epigenetic studies showed the testicular cell suspension group as the best group, this group is regarded the most similar to testes' micro-environment. The results of our on other culture groups, such as Sertoli and nanofiber, which kept the nature of human SSCs also confirms the importance of presence of ECM, micro-environment, and their signaling.

CONCLUSIONS

Despite the scientific development in stem cells, human SSC culture is still a controversial issue. Although our study has significant limitations due to the combined use of two-dimensional in vitro culture system, our results indicate that co-culture of human SSCs with testicular cells improved the germ cell ratio in vitro and could maintain a cell-specific genetic and epigenetic content. Thus, the results indicate the ability of proliferation of functional cells in suspension culture. However, further research is necessary for the evaluation of long-term human SSCs proliferation in vitro models. Recent reports have examined 3D in-vitro systems as an alternative to this study's 2D in-vitro culture model, which can mimic the natural niche of the SSCs and pave the way for in vitro spermatogenesis. Testicular cell culture in three-dimensional systems could be used as an

alternative way of restoring infertile patients' fertility. The results of the present study can dynamically add to the knowledge of researchers and clinicians and be an important step towards future clinical use for male infertility.

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CONFLICT ON INTEREST

Authors declare that there are no financial or conflict of interest exist.

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