Original Article

Genetic and Epigenetic Evaluation of Human Spermatogonial Stem Cells Isolated by MACS in Different Two and Three-Dimensional Culture Systems

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Abstract

Objective: Epigenetic and genetic changes have important roles in stem cell achievements. Accordingly, the aim of this study is the evaluation of the epigenetic and genetic alterations of different culture systems, considering their efficacy in propagating human spermatogonial stem cells isolated by magnetic-activated cell sorting (MACS).

Materials and Methods: IIn this experimental study, obstructive azoospermia (OA) patient-derived spermatogonial cells were divided into two groups. The MACS enriched and non-enriched spermatogonial stem cells (SSCs) were cultured in the control and treated groups; co-culture of SSCs with Sertoli cells of men with OA, co-culture of SSCs with healthy Sertoli cells of fertile men, the culture of SSCs on PLA nanofiber and culture of testicular cell suspension. Gene-specific methylation by MSP, expression of pluripotency (*NANOG, C-MYC* and *OCT-4*), and germ cells specific genes (*Integrin α6, Integrin β1, PLZF*) evaluated. Cultured SSCs from the optimized group were transplanted into the recipient azoospermic mouse.

Results: The use of MACS for the purification of human stem cells was effective at about 69% with the culture of the testicular suspension, being the best culture system. Upon purification, the germ-specific gene expression was significantly higher in testicular cell suspension and treated groups (P≤0.05). During the culture time, gene-specific methylation patterns of the examined genes did not show any changes. Our data from transplantation indicated the homing of the donor-derived cells and the presence of human functional sperm.

Conclusion: Our *in vivo* and *in vitro* results confirmed that culture of testicular cell suspension and selection of spermatogonial cells could be effective ways for purification and enrichment of the functional human spermatogonial cells. The epigenetic patterns showed that the specific methylation of the evaluated genes at this stage remained constant with no alteration throughout the entire culture systems over time.

Keywords: Azoospermia, Genetic and Epigenetic, Spermatogonial Stem Cells

Introduction

Male infertility is a disorder with complex and multifactorial etiology that caused researchers have been trying to achieve *in vitro* spermatogenesis (IVS) for a century (1). Epigenetic and genetic modifications roles are essential in spermatogenesis and embryogenesis of clinical approaches (2). *In vitro* culture and transplantation are two techniques of spermatogonial stem cells (SSCs) resuscitation after cryopreservation that could have a risk to change genetics and epigenetics (3). Consequently, SSCs grown *in vitro* due to exposure to growth factors and maturation processes can have a higher risk of becoming genetically modified. Therefore, special attention must be paid to the status of *in vitro* culture and post-transplantation (4).

A low number of SSCs about 0.03% of all germ cells in the rodents and no specific markers for identifying them have hampered rapid success in scientific development (5). Two major developments in SSCs culture include the establishment of the spermatogonial transplantation technique and the identification of glial cell derived neurotrophic factor (GDNF) as a key growth factor for the proliferation of SSCs *in vitro* (6).

Researchers have used different techniques for the proliferation of SSCs isolated from testis of azoospermic men, such as using human Sertoli cells as a monolayer in the absence of exogenous growth factors (7). Two-dimensional culture systems (2D) resulted in the incomplete proliferation and differentiation stages of SSCs. Thus, three-dimensional (3D) cultures have been

introduced very recently and have been hypothesized to be able to mimic seminiferous epithelium developing male germ cells better (3). Therefore, due to the improvement of culture conditions, one of the most widely used methods in tissue engineering is nanofibers. Poly(lactic acid) (PLA) is a form of an organic polymer obtained from lactic acid dissolved in water and carbon dioxide. Its most important properties are mechanical tensile strength, biocompatibility, and biodegradability. It contributes to mesenchymal stem cells and has chemical and mechanical properties similar to the extracellular matrix (8). In this study, the effect of culturing spermatogonia stem cells with a suspension of testicular cells, Sertoli cells, and culture on PLA nanofiber coated with laminin will be investigated.

Various techniques have been proposed for the isolation of very pure human SSCs (9, 10). Purification is suggested by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) (11). Panda et al. (12) used Ficoll gradient centrifugation chased by MACS and Thy1 surface marker for extraction of SSCs from growing L. rohita testis. Nevertheless, since there are enormous differences in spermatogenesis details between rodents and mammalians, further studies are essential, especially on humans.

In this study, for the first time, the genetic and epigenetic data on the effect of different *in vivo* and *in vitro* conditions evaluated. We aimed to examine genetic and epigenetic changes of human SSCs isolated by MACS and *GFRα-1* marker on their proliferation and purification capacity in various cultural systems. SSCs of azoospermic men who had obstructive azoospermia (OA) were cultured in five experimental groups; as a control group, with healthy Sertoli cells of fertile men, with Sertoli cells from men with OA, 3D culture system by PLA nanofiber and suspension groups evaluated. Finally, the SSCs function of the selected culture system after xenograft was considered to evaluate the effect of the length and nature of the culture system on the methylation pattern by the Methylation-specific polymerase chain reaction (PCR) (MSP) method.

Material and Methods

Sample collection

In this experimental study, human research specimens for four experimental groups were taken from men with OA via the intra-cytoplasmic sperm injection (ICSI) program from Shayanmehr Clinic (Tehran, Iran), whose remaining tissue was used for this study. Testicular samples from a fertile man who had an orchidectomy for reasons other than testicular problems were used to obtain healthy Sertoli cells. Testicular samples and the experimental procedure were authorized by Tarbiat Modares University's National Research Council guidelines (Tehran, Iran). The study goals were clarified to the contributors, and informed consent was taken from patients willing to take part in the research (52/12037). Inclusion criteria for a patient to enter the research are FSH levels between 15-1mIU/

ml, testicular volume 6-15 ml, dynamic biopsy with motile sperm, and pathology assessment is positive for spermatogenic cells (13).

Spermatogonial stem cells isolation and *in vitro* culture

Ten azoospermic men testicular biopsy samples for each experimental group were used and the biopsies specimens were transferred to the laboratory in a medium, within 60 minutes. They were broken into small pieces and placed in Dulbecco's Modified Eagle medium (DMEM, Gibco, Paisley, UK), got in touch with 14 mm $NaHCO_3$ (Sigma, St Louis, MO, USA), non-essential amino acids, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The broken pieces of the testis were placed in DMEM, which included 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. The gradient of gravity caused the spermatogenic tubules and cells to sediment. After three DMEM washes and the elimination of most interstitial cells, the next digestion step (45 minutes at 37°C) was done in by adding fresh enzymes and media to the fragments of the seminiferous tubule. With centrifugation at 1500 rpm for 4 minutes at 37°C, the cells were separated from the remaining tubule fragments.

Finally, the suspension of testicular cells was incubated in DMEM with FCS 10% and cultured overnight at 37°C and 5% CO_2. Sertoli cells stuck to the bottom of the container faster. In this way, after this period, the top cell suspension containing more germ cells and SSCs was collected.

In the first stage, a total of $2×10^5$ cells were cultured per 12-well plate in five groups for two weeks. You can see the results in our previous study (13). After enrichment of SSCs to enough count, suitable conditions were provided for SSCs purification with MACS. Then we cultured SSCs with and without MACS isolation for one week. Therefore, the cells of each experimental group were divided into two groups part was cultured as before for another week, and part was purified by MACS:

1. Control group, SSCs cultured in the culture dish.

2. Culture of SSCs with men's own Sertoli cells (Sertoli cells of men with OA). A testicular biopsied specimen was cultured at 37°C after digestion of the second enzymatic period. After 24 hours, the supernatant was removed and the bottom of the dish containing Sertoli cells was cultured with men's own sorted SSCs.

3. Culture of SSCs with normal Sertoli cells. To provide Sertoli cells for this group, a testicular sample from a fertile man who had an orchidectomy for reasons other than testicular issues was used to achieve healthy Sertoli cells. A testicular specimen was cultured at 37 °C after digestion of the second enzymatic period. After 24 hours, the supernatant was removed and the bottom of the dish containing Sertoli cells was cultured with SSCs (13).

4. Culture of SSCs on PLA nanofiber, covered with Laminin.

5. The suspension of biopsied testicular cells from biopsy cultured without separation of SSCs.

Three repetitions of each experimental group were cultured in 34-StemPro for one week with its complement (Invitrogen), 25 μg/ml human insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml –D (±) glucose, 30 μg/ml Pyruvic acid, 1 μ/ml –DL lactic acid, 5 mg/ml bovine serum albumin, 2 μM L-glutamine, -25×10^{-5} Mercaptoethanol, MEM soluble vitamins, 10^{-4} Ascorbic acid, 10 μg/ml -d biotin, 30 ng/ml beta-estradiol, 60 ng/ml Progesterone, 20 ng/ml human epidermal growth factor, 10 ng/ml derived neurotrophic factor class of glial cell and Humanities (GDNF), 10 ng/ml human leukemia inhibitory factor (LIF), 10 ng/ml basic fibroblast growth factor (bFGF), 5% FCS, 100 IU/ml penicillin, 100 μg/ml Streptomycin (Sigma), and the cells incubated at 37°C with 5% CO_2. We changed the cell culture medium every two days.

Purification of spermatogonial stem cells by MACS

To purify cultured cells, the cells in the plates were washed once with MACS buffer and then an appropriate volume of MACS buffer (based on the plate's level, number of cells, and the manufacturer's guideline) was added, and cells were mechanically isolated from the base by a cell scraper and centrifuged. After cell counting, cells were cultured in two groups with and without purification. In the second group, 200 μL buffer was added for all $2×10^6$ cells, and antibodies against GFRα-1 (SC-10716) diluted in 1:50 were added to the cells. Cells were refrigerated for 15 minutes. The column type was chosen based on the number of cells. The column was placed in a separator magnetic field. After washing the column and diluting buffer cells, secondary antibodies attached to Microbead (Milteny Biotec) diluted in 1:10 were added and incubated for half an hour at 4°C on the shaker. After washing with buffer twice and centrifuge, cells were labeled and poured into columns with buffer. Negative cells crossed the column while the GFRα-1 positive cells remained attached.

PLA nanofiber and Laminin preparation

The PLA fibers were made sterile by sinking in ethanol 70% for 2 hours or ultraviolet (14) radiation. Then, 20 μg/mL laminin (Sigma-Aldrich, USA) was poured on them and incubated at 37°C for 2 hours until one night. Before use, it was rinsed with phosphate buffer solution (PBS) and prepared for cell culture. Spermatogonial cell suspension prepared after one-night incubation of the second enzyme lysis was used in this group. The cell load was as follows: first, the cells were suspended in 30 μl of culture medium and a concentration of $3.5×10^5$ cells/mL. The fiber was added to the fiber dropwise and put in an incubator. After two hours, the rest of the culture medium was added. The cell medium was changed every other day.

Spermatogonial and sertoli cells confirmation

The Sertoli cells and SSCs were evaluated for vimentin and GFRα1 markers by immunocytochemistry. The cells were treated with an anti-vimentin antibody. The cells were fixed with 4% formaldehyde and became permeable by 0.2% Triton X100 and clogging with 10% goat serum (Vector, Burlingame, CA) for 30 minutes. The utilized primary antibody (mouse monoclonal anti-vimentin antibody with a dilution, 1:200; Sigma Company, USA) and the rabbit anti-human GFRα1 antibody (dilution 1:100) were added at 4°C and the dishes were incubated 24 hours. The fluorescent-labeled second antibody (1:100, Sigma) was added and incubated for 2 hours at 4°C in darkness. The cells were finally mounted with a mounting medium (Vector Laboratories Inc., Burlingame, CA) after three washes with PBS and examined under a fluorescence microscope (IX-71, Olympus).

Quantitative analysis of gene expression

Total RNA was isolated from SSCs derived from all groups, using an RNX-Plus TM (Cinnagen, Iran). RNA concentrations were evaluated by a UV spectrophotometer (Eppendorf, Germany). RevertAidTM first-strand cDNA synthesis kit (Fermentase) with oligo dT primer was used for reverse-transcription of treated RNA. 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.

The Thermal Cycler used SYBR Green and PCR master mix (Cinnagen) for PCR reactions (Applied Biosystems, StepOne TM, USA). Cycling conditions were initiated with a melting period at 95°C for 5 minutes, chased by 40 cycles of melting 30 seconds at 95°C, annealing 30 seconds at 58-60°C and extending 30 seconds at 72°C. Melt curve analysis was performed, and the standard curve for each gene was prepared using serial cDNA dilution from the testis to determine the output. The same run amplified the target gene and the reference gene. The ratio of gene expression was determined using the comparative cycle threshold (CT) method (n=3).

Epigenetic assessment

DNA extraction of spermatogonial stem cells

DNAs of the SSCs in all groups were extracted using a DNA extraction kit (Roche Co) based on the suggested guideline at the end of the culture. 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 at 70°C. Then, 100 μL isopropanol was added, then centrifuged after being transformed into a filtered tube. Finally, 50 μL elution buffer was added after centrifuge. The quality of DNA extraction was tested on agarose gel by absorption at 260-280 nm wavelengths.

DNA methylation by SSS1 enzyme

SSS1 methylase enzyme (Biolabs Co, New England) was used to guide the methylate primers to DNA, according to the instructions. After treatment with sodium

bisulfate (SBS), methylated primers were used for PCR. Enzyme stock 32 mmol was converted to 1600 μmol and incubated at 37°C for 1.5 hours. Heating up to 65°C for 20 minutes would stop the reaction. Then, methylated DNA was treated with SBS, before methylation-specific PCR (MSP) with M primer. Finally, methylated DNA was used as a positive control in MSP with methylated M primer.

Methylation-specific PCR

MSP was done by M primer with methylated DNA-modified sequence with SBS, and U primer with non-methylated DNA-modified sequence with SBS. Amplification with M primer showed methylation in CpG zones inside primer sequences, amplification with U primer showed no methylation, and amplification with both primers showed partial methylation in CpG zones inside primer sequences. In the present research, MSP with methylated and unmethylated primers was performed on *Integrin α6, Integrin β1, PLZF, C-MYC,* and *OCT-4.*

Spermatogonial stem cells labeling and transplantation

To confirm spermatogonial cell identity and function in spermatogenesis, SSCs resulting from cultures were transplanted to twelve NMRI mice, aged 6-8 weeks, with a mean weight of 25 g, kept in separate cages in fair conditions. Initially, the innate spermatogenesis was stopped by an intra-peritoneal injection of 40 mg/kg busulfan. After 4 weeks, the mice were azoospermic. For developing the azoospermia model, 40 mg/kg (Sigma, USA) of Busulfan was injected into each mouse for 4 weeks (15).

To detect and monitor the transplanted cells, spermatogonial colonies were mechanically extracted from culture plates under a reverse microscope. Then, after rinsing with PBS, they were exposed to Dil dye (2 μg Dil per 1 mL of PBS) for 5 minutes at room temperature. Next, they were kept at 4°C in darkness for 20 minutes. After confirming the cell dye under a fluorescent microscope, and three times rinsing in a culture medium, they were transplanted to the recipient mice. The technique used was similar to Brinster's technique (16). Before transplantation, the recipient mice anesthesia was performed through intraperitoneal injection of 10% ketamine and 20% xylazine. Then, the prepared SSCs were diluted at 10^5 cells per testis in a 10 μL culture medium (17) and micro-injected by 30 Gauge under stereomicroscope guide (Olympus SZ1145, Japan) at a concentration of $10^5/10$ μL DMEM to rete testis and finally to the left seminiferous tubule of the mice. To ensure the entrance of the cells to rete testis, 10% trypan-blue dye was simultaneously injected which makes the path visible to the naked eye. The right testis was chosen as the control. After 4, 8, and 16 weeks, the transplanted recipient animals were sacrificed and their testes were separated for assessments under a fluorescence microscope. The cell showing DiI staining was considered transplanted cells. The right testis is considered as the control group without transplantation of donor cells. Also, right side pictures are as phase contrast.

Assessment of the mouse testes' weight and epididymis sperms

The weight of testes was measured by an accurate digital scale after 16 weeks of transplantation in azoospermic mice (n=3). After separating the epididymis of both sides, they were placed in phosphate buffer solution with a pH of 7.4 which had been normalized in the incubator previously. They were then cut for the sperm to exit the epididymis. The phosphate buffer solution containing epididymis parts was incubated at 37°C and 5% CO_2 pressure for 30-45 minutes to increase the capacitated nature of the sperms. Finally, the number of sperms was counted by a hemocytometer under a light microscope and compared between the transplanted and control side.

Histological studies

For this part, samples were fixed in Bouin's fixative solution (Sigma-Aldrich, USA), dewatered by 70-100% alcohol, and elucidated by xylene twice. Then, the samples were dipped with paraffin twice for 1.5 hours, sectioned by microtome with a type C fixed blade (Leitz, Germany), and stained with hematoxylin-eosin (Merck, Germany). The technique was performed in accordance with the manufacturer's instructions (n=3 per condition). A total of 50 seminiferous tubes were chosen and the number of germ cells (spermatogonia, spermatocyte, and spermatid), as well as the number of cells per surface, were counted in 15 random fields with ×400 magnification under a light microscope.

Human *CatSper* expression gene in transplanted mice

To confirm the presence of human SSCs, quantitative PCR was used to detect human *CatSper* gene expression in cDNA of the transplanted testis of the mice after 16 weeks (n=3).

Statistical analysis

The statistical software SPSS version 16.0 for windows (SPSS Inc., Chicago, IL) was used and graphs were prepared by Microsoft Excel software version 2010. To analyze the data of real-time PCR, first, the raw data were converted to reportable data through available formulas and then analyzed by One-way ANOVA. P values of 0.05 are considered significant.

## Results

Confirmation of spermatogonia cells

The immunohistochemical staining of the isolated Sertoli cells obtained from the seminiferous tubules of testes biopsies of azoospermic men who had OA contained mostly two different cell types: First Sertoli cells which proliferated and formed a monolayer of cells as a feeder layer defined by vimentin. It was observed in the cytoplasm of the Sertoli cells (Fig.1A) around the nucleus (Fig.1B) and merged (Fig.1C). The second type with a spherical outline and two or three exocentric nuclei were spermatogonial cells creating colonies after proliferation (Fig.1D). *GFRα-1*, which is a spermatogonial stem cell nuclear marker, was found in the obtained colonies (n=3, Fig.1E).
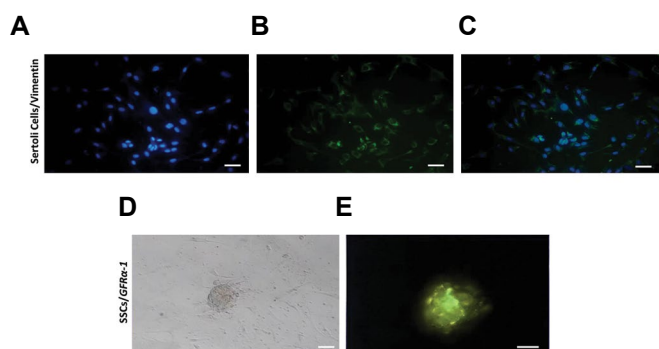
Fig.1: Spermatogonial and Sertoli cells confirmation. **A-C.** The immunohistochemical staining of the isolated Sertoli cells obtained from the human testes with detected Vimentin. **D.** Human spermatogonial stem cell colony. **E.** Immunofluorescent staining of spermatogonial stem cells (SSCs), detected *GFRα1* positive under an immune fluorescence microscope (scale bar: 200 μm).

Isolation of spermatogonial stem cells by MACS

After two phases of crossing the column (repeat separation), Figure 2 shows the immunohistochemistry of expression of GFRα-1 before and after cell isolation with MACS (Fig.2A, a-d). The percentage of GFRα-1-positive cells by the second isolation phase fraction of MACS was significantly higher than the percentage of positive cells after one isolation phase and before isolation (respectively, $69.01 \pm 3.54\%$ to $58.14 \pm 2.26\%$ and 37.7 ± 1.53, P<0.05, Fig.2.B).
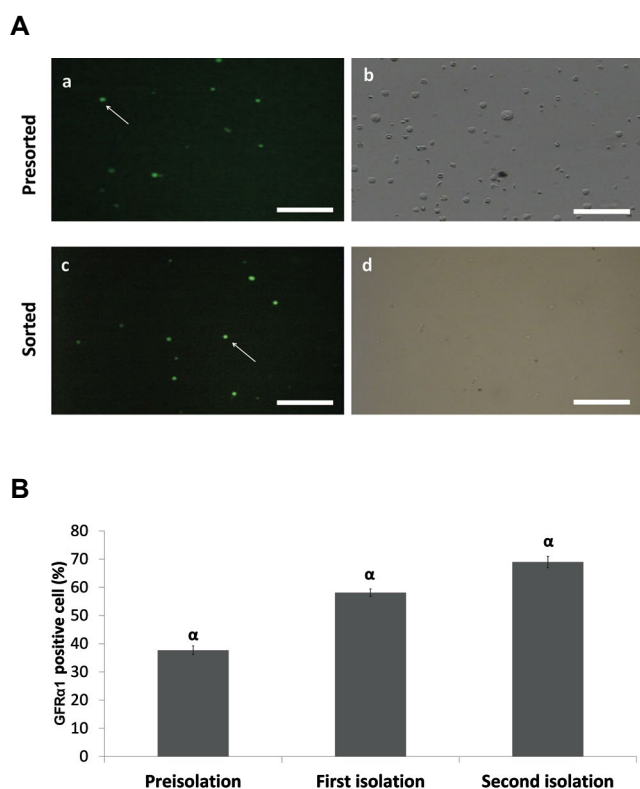
Fig.2: Isolation of spermatogonial stem cells (SSCs) by MACS. **A.** GFRα-1 immunohistochemistry to evaluate the expression of markers of spermatogonial cells before (a and b) and after (c and d) cell isolation by MACS. **B.** Expression of GFRα-1 marker of human spermatogonial stem cells derived from cultures after the two-step flowcytometry by MACS. α; Significant differences with the first time (mean ± SD, n=3, P<0.05) (scalr bar: 400 μm).

Results of quantitative polymerase chain reaction

Integrin α6, *Integrin β1*, and *PLZF* gene

Without MACS isolation, *Integrin-α6, Integrin β1*, and *PLZF* genes expression were significantly higher in testicular suspension cells than in other groups during culture (P<0.05). After cell isolation, the highest *Integrin α6, Integrin β1,* and *PLZF* expressions were observed in testicular suspension cells (P<0.05). Also, a comparison of cells isolated with and without MACS showed significantly higher expression of these genes in the isolated groups (P<0.05, Fig.3A).

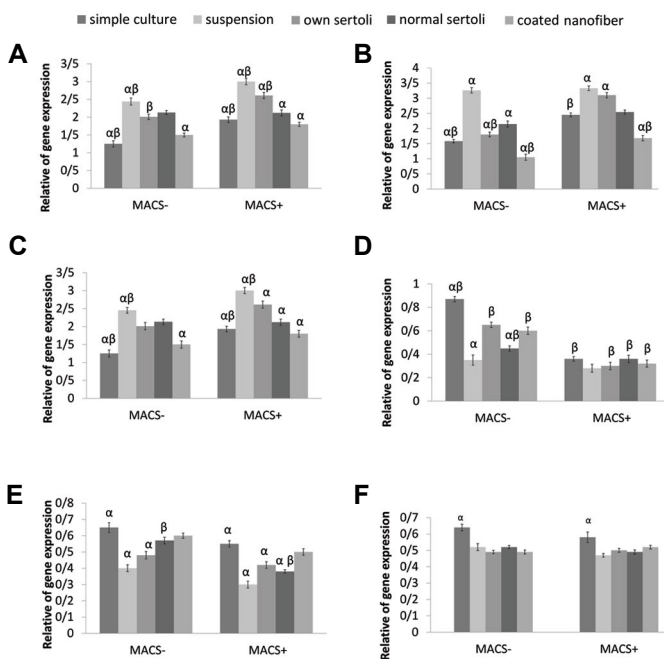### *NANOG, C-MYC,* and *OCT-4* gene expression

The expression of the *C-MYC* gene was lowest in the testicular suspension cells group without isolation of MACS than in healthy Sertoli, simple culture, and nanofiber after one week (P<0.05), but this difference was not statistically significant from men's own Sertoli cells group (P>0.05). After MACS isolation, it was significantly lowest in testicular suspension cells (P<0.05). Also, a comparison of cells isolated with and without MACS showed lower expression in the isolated groups (P<0.05).

The expression of Nanog after one week, without isolation of MACS, was lowest in testicular suspension and highest in simple culture (P<0.05), but this difference was not statistically significant between the men's own Sertoli cells group and nanofiber groups (P>0.05). After isolation with MACS, the expression of NANOG was lowest in testicular suspension and highest in the control group (P>0.05). The gene expression was lower in isolated than in non-isolated groups.

OCT-4 gene expression was highest in the simple culture, with and without cell isolation (P<0.05). The comparison of cells isolated with and without MACS showed no difference in gene expression (P>0.05, Fig.3).

Epigenetic results of MSP in different groups during culture

MSP results with methylated primer for *Integrin α6, β1*, and *PLZF* gene in all cultured cells had a similar pattern in all culture systems and remained non-methylated. The size of the proliferation fragment for Integrin α6 for methylated primers was 100 bp and for non-methylated primers 101 bp. They were 203, and 205 bp for *Integrin β1* gene, while for *PLZF* gene they were 125, and 130 bp, respectively. Methylation pattern did not 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* (Fig.4).

legend: ■ simple culture ■ suspension ■ own sertoli ■ normal sertoli ■ coated nanofiber

Fig.3: Quantitative gene expression analysis by qRT-PCR. **A.** *Integrin α6*, **B.** *β1*, **C.** *PLZF*, **D.** *NANOG*, **E.** *C-MYC*, and **F.** *OCT-4*, during spermatogonial cells culture in the studied groups. In each group, the expression level of a gene in each sample is normalized to *TBP*, as an internal control. The level of expression of each sample is also calibrated to a calibrator (the cells derived from second enzymatic digestion). α; Significant differences with other groups each time (P≤0.05), β; Significant differences between MACS⁺ and MACS⁻ groups (n=3, P<0.05, P≤0.05).

The results of *in vivo* assessment

Regarding the culture results of the previous steps as well as epigenetic studies, the suspension culture group was considered the best group, and the resulting cells were selected for transplantation. The azoospermic status of the mice before transplantation was confirmed (Fig.5A).

Monitoring the transplanted cells

The results of the sections' assessment showed that after 4 weeks, the cells were placed at the base of seminiferous tubes (Fig.5B); after 8 weeks, the tracked cells were shown in the diameter of seminiferous tubes (Fig.5C); and after 16 weeks, some seminiferous tubes of the transplanted testis (Fig.5D, left side) contained spermatozoa. Also, endogenous spermatogenesis was observed in the control testis (right side, Fig.5B-D).

Comparison of the testes' weight and number of sperms in epididymis between the transplanted and control testis

The mean testis weight of the azoospermic SSCs transplanted and control mice showed a significantly higher mean weight than azoospermic (sham group: without SSCs transplantation) and the opposite side testis of the transplanted azoospermic mice that were not transplanted after 16 weeks (0.08 ± 0.00058, 0.095 ± 0.0015, 0.057 ± 0.0032, 0.063 ± 0.0051g respectively, P<0.05). The mean

testis weight of the transplanted group was significantly lower than the mean testis weight of normal healthy adult mice (P<0.05, Fig.6A).

Assessment of the number of sperms in epididymis revealed that the transplanted and control testis had significantly higher mean sperms than the sham and opposite side testis groups after 16 weeks (26 ± 5, 31 ± 9, 18.7 ± 4, 16.3 ± 5 respectively, P<0.05). The mean sperm count of the transplanted group was significantly higher than the mean sperm count of the sham and opposite side testis groups (P<0.05). The mean number of sperms in the control testis of the transplanted mouse was similar to the transplanted group (P>0.05, Fig.6B).
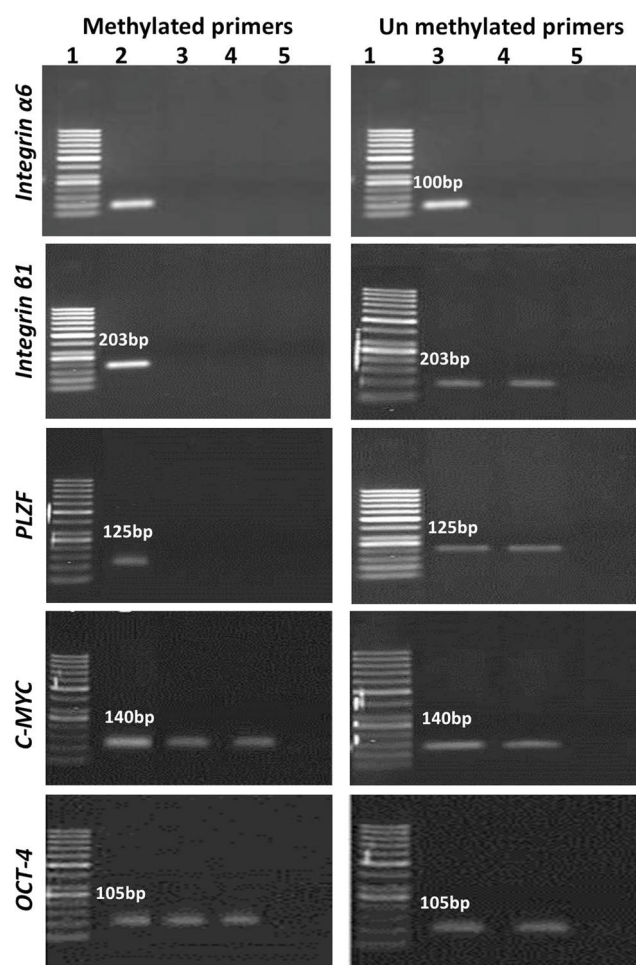

Fig.4: Results of MSP-PCR for methylation of genes *Integrin α6, β1, PLZF, C-MYC*, and *OCT-4*. Left image: MSP with methylated primer. Right Image: MSP with non-methylated primer. Column 1; Ladder 50 bp, Column 2; Positive control of methyl primer, Column 3; The group MACS⁻ one week after *in vitro* culture, Column 4; Group MACS⁺ one week after *in vitro* culture, and Column 5; Negative control.

Histopathology of testis sections in the transplanted and control testis

Based on Figure 6C-E, the mean number of spermatogonia, spermatocytes, and spermatids was higher in the transplanted testis than in the control and sham (P<0.05), but it was

significantly lower than that of normal healthy adult mice (P<0.05). The mean number of cells in the control testis of the transplanted mouse was similar to that of the sham group (P>0.05).
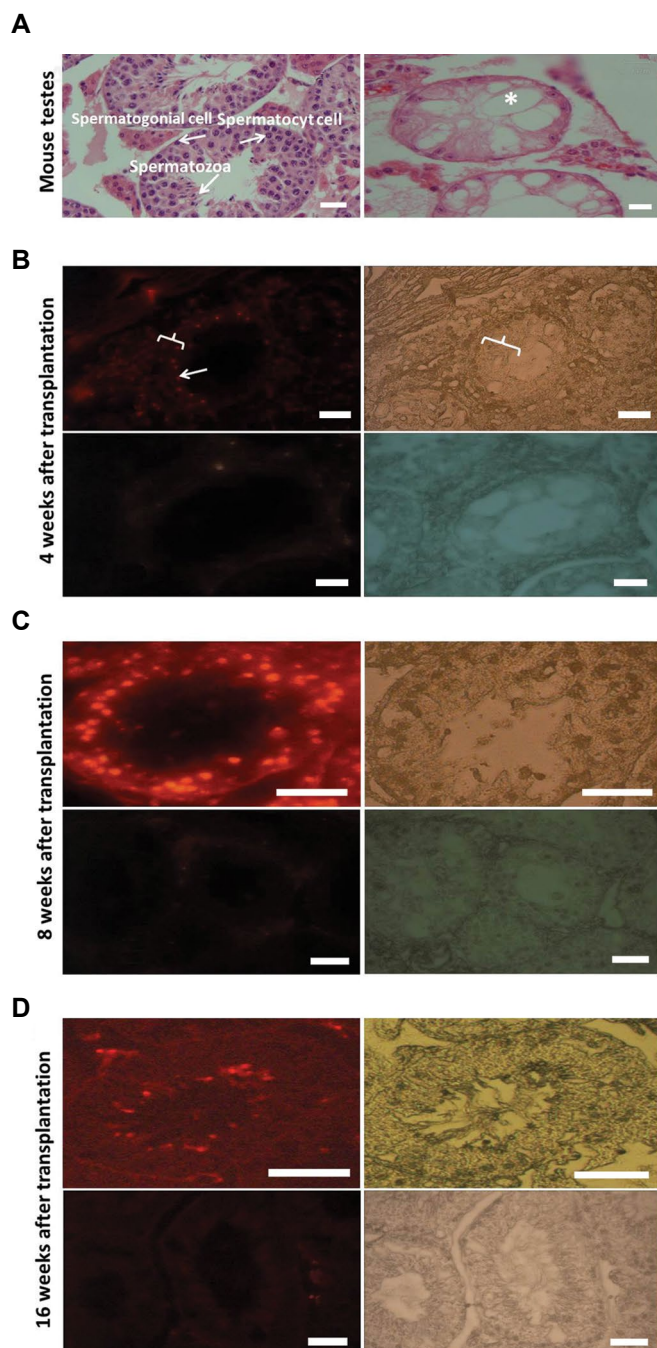
Fig.5: The results of *in vivo* assessment. **A.** Histological examination of seminiferous tubules in mouse testes before and after busulfan injection. **B-D.** Tracking human spermatogonial cells 4, 8, and 16 weeks after transplantation. **B.** Human SSCs on the base of the mouse seminiferous tubules after 4 weeks of transplantation. **C.** Human SSCs within the mouse seminiferous tubules 8 weeks after transplantation. **D.** Derived sperm were observed in the mouse testes 16 weeks after transplantation. The cell showing DiI staining was considered transplanted cells. The top figures in each group (A-D) are the sections of the testicular seminiferous tubules transplanted and the bottom figures are related to the opposite testicle as a control (the right testis is considered as the control group without transplantation of donor). The figures on the left side are fluorescent and the figures on the right side are phase contrast (scale bars: 20 μm).

Assessment of *CatSper* gene in the transplanted testis

CatSper gene was evaluated after 16 weeks of transplantation in the azoospermic mouse model. The expression of this gene in the transplanted testis showed proliferation and presence of the human *CatSper* gene in the transplanted testis. Nevertheless, the results showed significantly lower *CatSper* gene expression in the transplanted testis than in the human testis (P<0.05). The expression of this gene was zero in the control testis (Fig.6F).
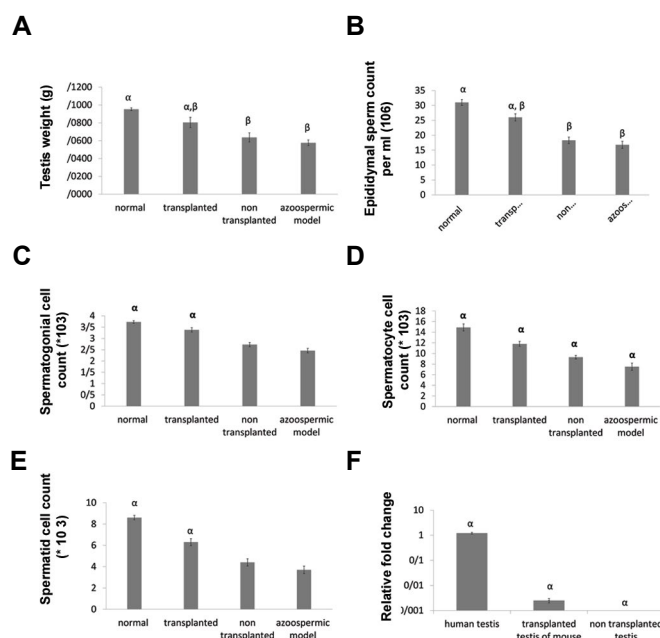
Fig.6: Comparison between the transplanted and control testis. **A.** Mean testis' weight (s), **B.** Mean the number of sperms in the epididymis among the groups. **C.** Mean the number of spermatogonia, **D.** Spermatocytes, **E.** Spermatids in seminiferous tubes among the study groups. **F.** The pattern of CatSper gene expression after 16 weeks of transplantation in the azoospermic mouse model. α and β; Significant differences with others (P≤0.05).

Discussion

Optimization of a system for the proliferation and differentiation of male germ cells is a valuable tool for managing male infertility and spermatogenesis regulation (18). One of the important regulators in different spermatogenesis processes is epigenetic modifications (19). Genetic and epigenetic structures of chromatin are essential for fertile sperm production (20). Hence, we used MACS for human SSCs purification by *GFR-α1* positive marker. *GFRα1* is a self-renewal-related or pre-meiotic gene, expressed in undifferentiated SSCs such as A_{single}, A paired and spermatogonia $A_{alelled}$ (21). The MACS isolated SSCs were cultured in different culture systems and compared for genetic and epigenetic expression between five experimental groups; i. Control two dimensional culture, ii. Co-culture of SSCs with

Sertoli cells of men with OA, iii. Co-culture of SSCs with healthy Sertoli, iv. Culture of SSCs on PLA nanofiber, and v. Culture of testicular cell suspension. Researchers have similarly investigated the isolation of human SSCs through the FACS method (9, 22). Meanwhile, the high costs and time-consuming nature of FACS may limit its application in cell isolation. Thus, we used two-step enzyme lysis and incubation of the resulting cells for one night and isolation of suspended spermatogonial cells on the next day, as required in different groups. The results of the present study confirmed *GFR-α1* (GDNF receptor) as an effective marker in improving SSCs isolation, which has been previously suggested by Godmann et al. (23) as well. It has been confirmed that GDNF supplies the necessary items for the growth and maintenance of human SSCs in the medium and generally shows the suitability of MACS with *GFR-α1* for isolation and enrichment of human SSCs (24). Miltenyi et al. (25) suggesting MACS as a fast and simple separation system for large immunologic cells. Baert et al. (26) cultured MACS-enriched epithelial cells in the interstitial cell-laden scaffolds (CD49f+/CLS). They observed double-cell compartment testicular constructs. Cell spheres showed in the pores after cell seeding on CFS and CLS. The elongated spermatids were observed in 66% of TC/CFS. Differentiation was achived in all and 33% of CD49f+/CLS constructs, respectively.

The MACS enriched and non-enriched (SSCs cultured without MACS sorting) SSCs were cultured in the control and treated groups; co-culture of SSCs with Sertoli cells of men with OA, co-culture of SSCs with healthy Sertoli cells, the culture of SSCs on PLA nanofiber, and culture of testicular cell suspension. We observed significantly the highest expression of *Integrin α6* and *PLZF* genes in the testicular suspension cells group and lowest expression in the simple culture group than in other groups. *Integrin β1* gene expression was highest in testicular suspension cells and lowest in the PLA nanofiber group, which confirmed that testicular suspension cells could effectively purify and enrich the functional human spermatogonial cells. *Integrin α6* and *Integrin β1* are premeiotic markers and they have a connection to laminin and collagen proteins in the base membrane of seminiferous tubes (6). Nevertheless, the expression of *OCT-4* gene was not significantly different among groups. The gene expression of *OCT-4* and *PLZF* is related to GDNF and affects the self-renewal of SSCs (27, 28). The three-dimensional culture, with the aid of an extracellular matrix, enables cells to organize properly and imitate the spermatogonial epithelium (29). The use of three-dimensional culture on PLA nanofiber with a laminin layer in the present study showed the superiority of this method over simple culture, regarding germ-cell specific gene expression. We used PLA, because of their biodegradability and biocompatibility, Which is quickly made by electrospinning and creates a three-dimensional non-woven grid. Eslahi et al. (8) cultured SSCs seeded on PLLA with the control groups and suggested that PLLA increases the colony formation of human SSCs in the culture system. Also in the present study, a superior co-culture with Sertoli cells in comparison with the control

group was observed. It might be SSCs culture with GDNF-secreting Sertoli cells acting as spermatogonial cell renewal regulator. Koruji et al. (30) co-cultured human SSCs and Sertoli cells and observed an increase in the count and diameter of SSC colonies. The gene expression of pluripotency genes showed minimum expression of *C-MYC* gene and Nanog in testicular suspension cells, especially after isolation with MACS with increased gene expression during culture in all isolated groups. Rajpert-De Meyts et al. (31) could isolate multivalent germ cells from the adult human testis by using suitable culture conditions to isolate embryonic stem cells. They observed that these cells express *OCT-4* proteins but do not express *NANOG*.

According to our previous study, the suspension culture group is regarded as most similar to testes' micro-environment (13). We observed that the other culture groups, such as Sertoli and PLA nanofiber, which kept the nature of human SSCs also confirms the importance of the presence of extracellular matrix, micro-environment, and their signaling. On the other hand, germ cells often show genetic and epigenetic changes *in vitro*, and SSCs seem to maintain relative genetic stability. SSCs characteristics did not change and cells were not differentiated suggesting the stability of this culture technique, which could be due to the protective effect of GDNF against differentiation (32). It can be a reason for prolonged epigenetic changes after culture among different groups with and without MACS. This may limit the assessment of gene expression and DNA methylation to only some printed genes. Because of this, comparative genomic hybridization is not able to detect small genetic changes. Goossens et al. (33) evaluated the DNA methylation pattern in a paternally methylated gene (*Igf2*), a maternally methylated gene (*Peg1*), and a non-imprinted gene (*α-Actin*). The spermatids obtained from the 3D-I system have similarities in global gene profile and DNA methylation compared to *in vivo* spermatids. They used MACS for human spermatogenesis *in-vitro*, by isolating GPR125+ spermatogonia from the testes of OA patients.

Transplantation of SSCs is a fertility restoration option that has already been introduced as a convenient method in animals (16). Studies on the imprinting situation after SSCs transplantation are limited but show that implantation does not change. The results of the present study on transplantation of human SSCs, cultured with healthy Sertoli cells to seminiferous tubes of a recipient mouse, showed that it can result in spermatogenesis with a donor origin. Although transplantation of human SSCs is not likely to produce sperm in mice, it may activate endogenous spermatogenesis-stimulating factors. Thus, we evaluated sperm production in mice after 16 weeks. When the SSCs from the donor are transplanted to the seminiferous tubes of an infertile recipient, the germ cells of the donor migrate to the lateral base of the tubes in the seminiferous tubes of the recipient, then proliferate, produce new colonies, and start spermatogenesis

with a donor origin (35). Although initial studies have experimented with mice models (16), this technique can be useful in spermatogenesis studies of other animals as well. Indeed, some studies have reported successful heterografts from mouse testis to hamsters that resulted in spermatogenesis of mouse and hamster (36). There are several studies that transplanted human SSCs into the mouse testis and reported that SSCs adhere to the seminiferous tubules after 2 weeks (22, 34). Mohaqiq et al. (34) isolated human SSCs and confirmed them by *PLZF* protein. They transplanted SSCs to adult azoospermia mouse testes and studied them after two weeks. The results revealed that the number of SSCs was significantly more than those in the control group. IHC studies and qRT-PCR indicated that the *PLZF* was only expressed in the transplantation groups. The results of SSCs transplantation in our study were similar to others (9, 34).

GCT, as the ultimate goal of these cellular studies, could successfully restore spermatogenesis in animal models and resolve infertility, which is considered the gold standard (37). Further research continues to elucidate different aspects of GCT for successful experiments on humans. Despite the scientific development in stem cells, human SSCs culture is still a controversial issue. The results of the present study can dynamically add to the knowledge of researchers and clinicians and is an important step toward future clinical use for male infertility, which was the strongest strength of the present study. In addition, we could successfully achieve an appropriate number of cells through a two-phase culture which was an important limitation in previous studies (38). Yet, the current study had some limitations, including the fact that the transplantation experimented on mice and thus, the results cannot easily be generalized to humans. Further research on mammals can add to the results of the present study.

Conclusion

The epigenetic pattern showed that the specific methylation of the evaluated genes at this stage remained constant throughout the entire culture system over time and the culture conditions did not alter the methylation pattern. Also, MACS could increase the efficiency of human SSCs isolation and purification by 69% with the testicular suspension group showing the highest expression of germ cell genes (*Integrin α6, β1*, and *PLZF*), and lowest gene expression of *C-MYC* gene and *NANOG*, among the tested groups. Further, the proposed culture systems could maintain the cell-specific genetic and epigenetic contain and the suspension cells group known as the best system for SSCs culture *in vitro*. Thus, the results indicate the ability of purification and proliferation of functional cells in the suspension culture.

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Authors' Contribution

M.Z; Performed the experiments, data acquisition, data analysis and interpretation, and drafting the manuscript. M.M.; Was the conductor of the study, participated in study design, edited the manuscript, also participated in the finalization of the manuscript, and also approved the final draft. S.J.M., M.N.; Advised for genetic and epigenetic assessment, real-time PCR technique, extracted mRNA and produced cDNA. M.R.N; Advised sample collection and separated SSC from samples. M.K., F.A.; Performed transplantation and participated in statistical analysis, and edited the manuscript. All authors were involved in the drafting and revision of the draft manuscript.

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