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In vitro spermatogenesis assessment after transplantation of human spermatogonial stem cells

Mahdi Mohaqiq[1], Mansoureh Movahedin[2], Zohreh Mazaheri[3], Naser Amirjannati[4]

1- Scientific board member of medicine faculty, Kateb University, Kabul, Afghanistan
(Corresponding Author) Mohaghegh_mahdi@kateb.edu.af
2- Scientific board member of medicine faculty, Tarbiat Modares University, Tehran, Iran.
movahed.m@modares.ac.ir
3- Assistant professor, Basic Medical Science Research Center, Histogenotech Company, Tehran, Iran. Zohremazaheri@yahoo.com
4- Associated professor, Department of Andrology and Embryology, Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.

Abstract

Background: The introduction of alternative systems in vivo can be very important for cancer patients who are treated with gonadotoxic methods and drugs. In this study, we examine the progression of the spermatogenesis process after transplantation of human spermatogonial stem cells (SSCs) under conditions of testicular organ culture and in vivo condition.

Materials and Methods: Human SSCs were obtained from TESE sample, and the nature of these cells was confirmed by detecting the PLZF protein. These cells, after being labeled with DiI, were transplanted to adult mice testes, treated with Busulfan 40 mg/kg as azoospermia model. Then the culture of host testicular tissue considered as a test group and in vivo transplant as a control group. After 8 weeks, immunohistochemical, morphometric and molecular studies were performed.

Results: The results of morphometric studies indicated that the mean number of spermatogonia, spermatocytes, and spermatids in test groups was significantly lower than the control group (P<0/05) and most of the cells responded positively to DiI tracing. Immunohistochemical in both groups revealed expression of the PLZF, TP1 and Tekt1 proteins in spermatogonial, spermatocyte and spermatozoa, respectively. Also, in human molecular studies PLZF, TP1 and Tekt1, the results showed a significant difference in the expressions of these genes between test groups and control groups (P<0/05).

Conclusion: These results suggest that the conditions of testicular tissue culture after transplantation of SSCs can support the development of spermatogenesis, as well as in vivo.

Key words: *stem cells, human, transplantation, culture.*

Introduction

Culture systems, that reproduce the male reproductive cells in the culture medium, are various [1]. This seems to be the case for shortening a complex process to smaller parts for experiments, manipulations, and understanding of it. Culture systems have existed at the cellular and molecular level [2]. Laboratory cultures have at least the permission to manipulate the paracrine environment and also to examine the effect of the role of each growth factor individually on the sperm production process [3]. The three dimensional (3-D) testicular tissue culture systems maintain the necessary conditions for the progression of the spermatogenesis due to the maintenance of the 3-D structure of the seminiferous tubules and interstitial tissue [4]. It seems that this system can be used to induce and resume the spermatogenesis by spermatogonial stem cell transplantation, to produce mature sperm and an application that is a high-level reproductive medicine target [5]. This issue is even more important in cancer patients who are exposed to chemotherapy and radiotherapy treatments because of the high risk of returning cells to cancer patients before treatment [6]. Reports on the potential of testicular tissue culture systems have recently begun to be published. Few studies have reported tissue culture optimization. Sato et al used immature mouse testicular tissue fragments to reach fully functional spermatozoa [7]. They isolated germ cell mice from testicular tissue and labeled with Green Fluorescent Protein (GFP) and transplanted into immature azoospermia testicular tissues by in vitro transplantation and then under tissue culture conditions. After 6 weeks, they reported a functional and adult sperm extraction that could be used for ART. In 2013, Yokonishi et al introduced the system of testicular tissue culture on the agarose gel with a report of sperm obtaining. They split the immature mouse testicular tissue into small pieces and place it on the agarose gel under culture conditions [8]. In 2010, Gobara et al also reported the release of round haploid spermatids by culturing of the testicular tissue on the agarose gel [9]. In another report, Sato et al put the immature mouse testis tissue that contains only gonocytes and spermatogonial cells (SCs) precursor under tissue culture conditions on agarose gel and, that leads to full progression of spermatogenesis. They obtained yielded adult mature sperm that can be fertilized by microinjection. They also placed immature mouse testicular tissue after freezing and thawing under tissue culture conditions and reported complete progress in spermatogenesis [10]. In the present study, human spermatogonial stem cells are transplanted in mature azoospermia testis and placed on agarose gel under 3-D tissue culture conditions. However, until now, the progress of the spermatogenesis process has not been studied by human SCs.

Materials and methods

Sample preparation and freezing-thawing protocol

Samples were obtained from the rest of the testicular tissue samples of azoospermia patients after the completion of the treatment. All stages of this research were based on the approval of the research ethics committee of Tarbiat Modares University with the registration ID IR.TMU.REC.1394.68. For the freezing and thawing of testicular tissues, the protocol of the Honaramooz et al [11] was used. In this way, the small parts of the testicular tissue were placed in a special freezing medium and then placed in a programmable freezer (Planner Cryo 360. 1/7- UK). The machine automatically processes all the freezing steps slowly and stepwise. Finally, the frozen tissue is ready to leave the machine and put in liquid nitrogen.

Testes biopsies were first scraped with scissors and scrapers, about half a centimeter, and then inside Leibovitz-L-15 (Gibco- UK) cryovials containing 2% FBS (Sigma- USA) and 10% DMSO (Sigma- USA). The tissues were incubated at room temperature for 15 minutes and then placed in a programmable freezer. The testicular tissues were cooled at -20 ° C to -4 ° C at -2 ° C / min. At this stage, seeding was done manually using a penny that was previously placed in liquid nitrogen. The cooling cooled from --4 ° to -30 ° C at -0.3 ° C / min and then cooled down from- 30 ° C to -130 ° C at -10 ° C / min. The vials were immersed in liquid nitrogen and then transferred to the storage chamber and held for 48 hours. To melt, the vials were kept at room temperature for 1 minute and then placed in a 25 ° C bath for 1 minute. 1 ml of Leibovitz L-15 medium was added to each vial and transferred to sterilized centrifuge tubes. To remove free radicals, the testes were washed twice with the Leibovitz L-15 medium.

Isolation, culture and confirmation of identification of spermatogonial stem cells

SSCs were isolated by Mirzapour et al protocol under two steps of enzymatic digestion with trypsin (0.5 mg/ml, Sigma, USA), collagenase (0.5 mg/ml, Sigma, USA) and DNase (0.05 mg/ml, Sigma, USA) enzymes [12]. Because of the present small number of SCs present in TESE biopsy, after digestion, to enrich and enhance the SCs, these cells were cultured for two weeks. The identification of isolated and purified SCs was investigated by tracing the *PLZF* protein as a stem cell marker in colonies derived from cell suspension.

Preparation of agarose support layer for tissue culture

To provide an agarose support layer we used the Yokonishi et al method [8]. The agarose solution 1.5% (Carl Roth, German) was prepared and then sterilized. At the time of using the 1×1×0.5 mm agarose components in culture containing about 4/5 of the α-MEM (Alpha Minimum Essential Medium) (Bio-Ideal, I.R.I) containing 10%

KSR (Knockout Serum Replacement) (Gibco, UK), progesterone (Invitrogen, UK) at a maximum concentration of 60 ng/ml, beta-estradiol (Pepro Tech, USA) maximum concentration 30 ng/ml and EGF (Epithelial Growth Factor) (Pepro Tech, USA) at a maximum concentration of 20 ng/ml, FGF (Fibroblast Growth Factor) (Pepro Tech, USA) maximum concentration of 10 ng / ml, GDNF (Human glial cell line-derived neurotropic factor) (Pepro Tech, USA) maximum concentration of 10 ng/ml and LIF (Leukemia Inhibitory Factor) (Royan, I.R.I) maximum concentration of 10 ng/ml. The tissue parts were gently placed in the middle of the agarose layer so that they did not float. The culture medium was changed twice a week.

In vitro transplantation of spermatogonial stem cells to the testes

To detect the transplanted cells and purify them from endogenous cells, the cells cultured with a density of about 80% before transplantation for 5 minutes exposed to a 2 µg DiI color (Eugene.OR, USA) from a 1 ml preservative solution PBS was placed at room temperature and then placed in a dark place for 20 minutes at 4 ° C. After ensuring that the cells were stained under a fluorescent microscope, the cell surface was washed with PBS and then isolated from of petri dish by trypsin enzyme (25%) in 0.1% EDTA (Sigma, USA). after 3 times washing in the medium, they were transplanted into the host testis. To stimulate the mouse azoospermia model, 40 mg / kg dose of busulfan was used. This caused after 4 weeks, adult mouse testes to be virtually empty of spermatogenesis. Spermatogonial stem cells were transplanted into the host testes below the stereo microscope was cut into small pieces and placed under 3-D tissue culture conditions on the agarose support layer. For in vitro transplantation of SSCs to exited host testes we used Sato et al protocol [10]. In this way, using a glassy needle, entered to efferent ductuli and injected cells to the end of the efferent ductuli and the early of ret of testis (Fig. 1). A 10 µL cell suspension containing 100000 cells was spread into seminiferous tubules and filled about 40-80% of testis.

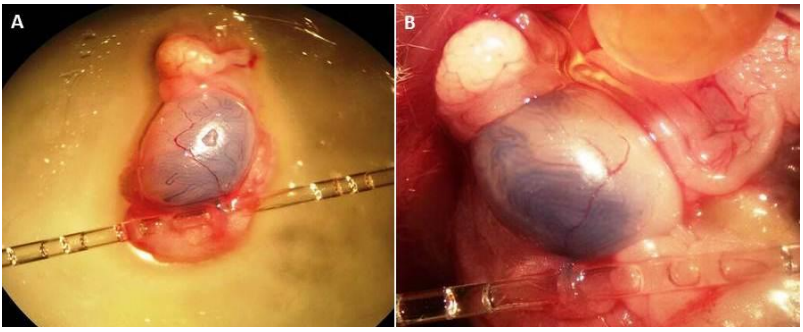

Figure 1. Transplantation of SSCs to host testes. Transplanted SSCs were entered to efferent ductuli and then conducted to seminiferous tubules. A: IVT. B: In vivo transplantation

Morphometric evaluations of the host testes

An optical microscope equipped with an ophthalmologic eye lens was used to measure the various structural parameters in the sections prepared from the host testes [2]. A total of 5 sections, spaced equally apart, were selected from successive sections of each testis. After staining of each section, 10 seminiferous tubules with rounded or close-circle sections randomly selected were used to evaluate the testicular parameters. Therefore, 50 seminiferous tubules were selected from each group. The number of SCs, spermatocyte and spermatid per unit volume were measured in each testis. Two histological sections were prepared from each recipient testis with an interval of 12 μm to obtain the percent of tubules with SSCs subsiding on the seminiferous tubules.

Immunohistochemical studies

The immunohistochemistry method was used to prove the intrinsic nature of the cells claimed to be germ cell types. For this purpose, the testicular tissue fragments of the experimental groups. Besides, the tracing of DiI were subjected to immunohistochemistry after tissue processing. To confirm the nature of spermatogonial stem cells from Oct-4 antibodies [13], the spermatocyte from the Tekt1 antibody [14] and Spermatozoa were used for the Tnp1 antibody [15]. The procedure of immunocytochemistry was performed according to the previous study8. Briefly, the cells were grown on the glass slides and fixed for 20 minutes in 4% paraformaldehyde at room temperature, before rinsing with PBS. After permeabilization by 0.2% Triton X-100 (MP Biomedicals, USA) for 1 hour to facilitate antibody penetration, the slides were washed with PBS supplemented with 0.2% bovine serum albumin. Nonspecific antigens were blocked with 10% normal goat serum (Vector Laboratories, USA). The slides were then incubated overnight at 37ºC with a mouse monoclonal antibody. The slides were washed with PBS and then the second antibody was applied for 2 hours at room temperature in the dark.

Molecular studies using Real-Time PCR

To prove the presence of different classes of germ cells and to prove that these cells are not due to intrinsic spermatogenesis of the mouse testis, testicular fragments of the experimental groups were studied in the of Oct-4, Tekt1 and Tnp1 genes. The protocols of Real-Time PCR were done based on the previous study [16]. The humanity of primers designed to differentiate cells of the murine nature with germ cells of human nature.

Design, order and prepare primers

To design the primers used in Real-Time PCR, the gene sequences from Oct-4, Tekt1 and TP1 were obtained from the NCBI database and the sequence of their exons and introns was determined. Primer design was done using the Primer3 Online Software. Real-time PCR primers were designed to have at least one of the Forward or Reverse primers in the interconnection region between the two exons to prevent possible contamination of the genomic DNA in Real-Time PCR. Designed primers are blasted to confirm their accuracy and reproduce only the genes' mRNA sequences. The sequences of the Real-Time PCR primers of Oct-4, Tekt1, and TP1 genes are shown in Table 1.

Data analysis

In this study, all quantitative data were presented as mean ± standard deviation. One-way ANOVA, T-test, and Tukey test were used for statistical analysis. The significance level was considered to be $P<0.05$.

Results

The TESE biopsies were subjected to two stages of enzymatic digestion. First, the preparation steps, and second, a cell suspension containing SCs was obtained under culture conditions. The identification of the SSCs was confirmed by tracing the *PLZF* protein in the colonies derived from the cultured cell suspensions obtained (Fig. 2).

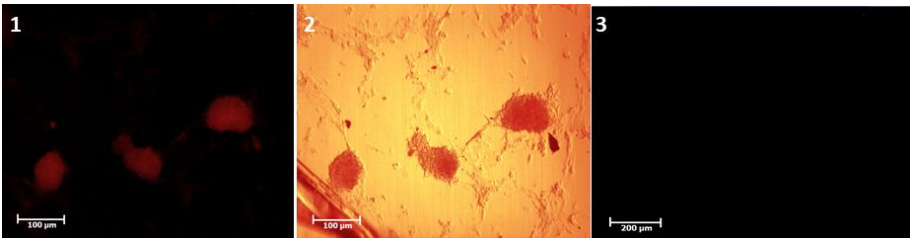

Figure 2. Confirmation of the nature of spermatogonial stem cells. Expression of PLZF protein in colonies obtained from culture of human SCs (1). Cluster contrast image (2), negative control group without primary antibody (3).

8 weeks after transplantation, the groups were evaluated. The histological studies results showed a progression of spermatogenesis and complete repair of epithelium of seminiferous tubules in both groups but it was shown that in the in vivo group the reparation of epithelium and form of seminiferous tubules was better than IVT group (Fig. 3). The results of immunohistochemistry of host testes in both groups showed

that the repaired epithelium cells express positively *PLZF*, *SCP3* and *ACRBP* proteins in the immunohistochemical studies, which are the specific proteins of SSCs, spermatocytes and spermatozoa, respectively (Fig. 5-7). The results of the morphometric studies were that the number of SCs, spermatocytes and long spermatids, or sperm cells, as well as the percentage of seminiferous tubules with epithelium in the in vivo group was higher than the in vitro group significantly ($P<0.05$) (charts 1). The number of SCs in the in vivo and IVT groups were ($220\times103 \pm 8.4\times103$) and ($183\times103 \pm 15\times103$) respectively that shown the significant high number of SCs in the in vivo group ($P<0.05$). In the next, the number of spermatocytes in the in vivo and IVT groups were ($407\times103 \pm 18\times103$) and ($341\times103 \pm 20\times103$) respectively that showed the significant high number of spermatocytes in the in vivo group ($P<0.05$). Finally, the number of long spermatid in the in vivo and IVT groups were ($416\times103 \pm 2\times103$) and ($256\times103 \pm 5\times103$) respectively that showed the significant high number of long spermatids in the in vivo group ($P<0.05$). Also, the percent of repaired seminiferous tubules were shown a significant difference between groups (Chart 2). The percent of tubules with epithelium in the in vivo and IVT groups were (96%± 3.2%) and (83.3%± 5.2%) respectively, that is shown the significant difference between groups ($P<0.05$).

The results of host testes Real-Time PCR studies were shown the expression of *PLZF*, *Tekt1*, and *Tnp1*genes, respectively, of SSCs, spermatocytes and spermatids or spermatozoa were expressed positively in the in both groups without significant difference ($P>0.05$) (Chart 3).

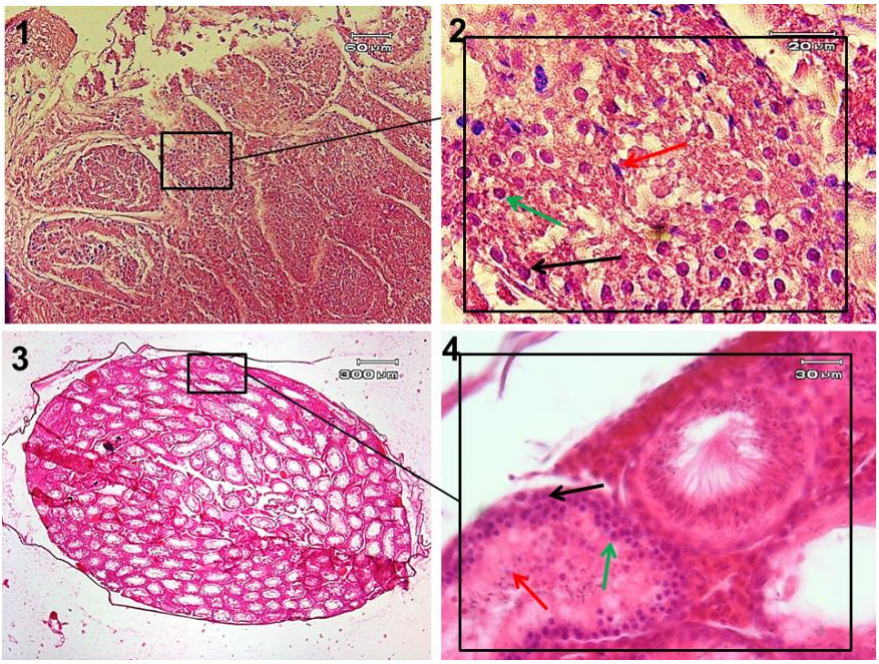

Figure 3. H & E staining of tissue sections IVT and in vivo transplantation groups. Tissue section of IVT group (1 and 2). Tissue section of in vivo transplantation group (3 and 4). Black arrow suggested: SCs, green arrow suggested: spermatocyte and red arrow suggested: Long spermatid or sperm cells

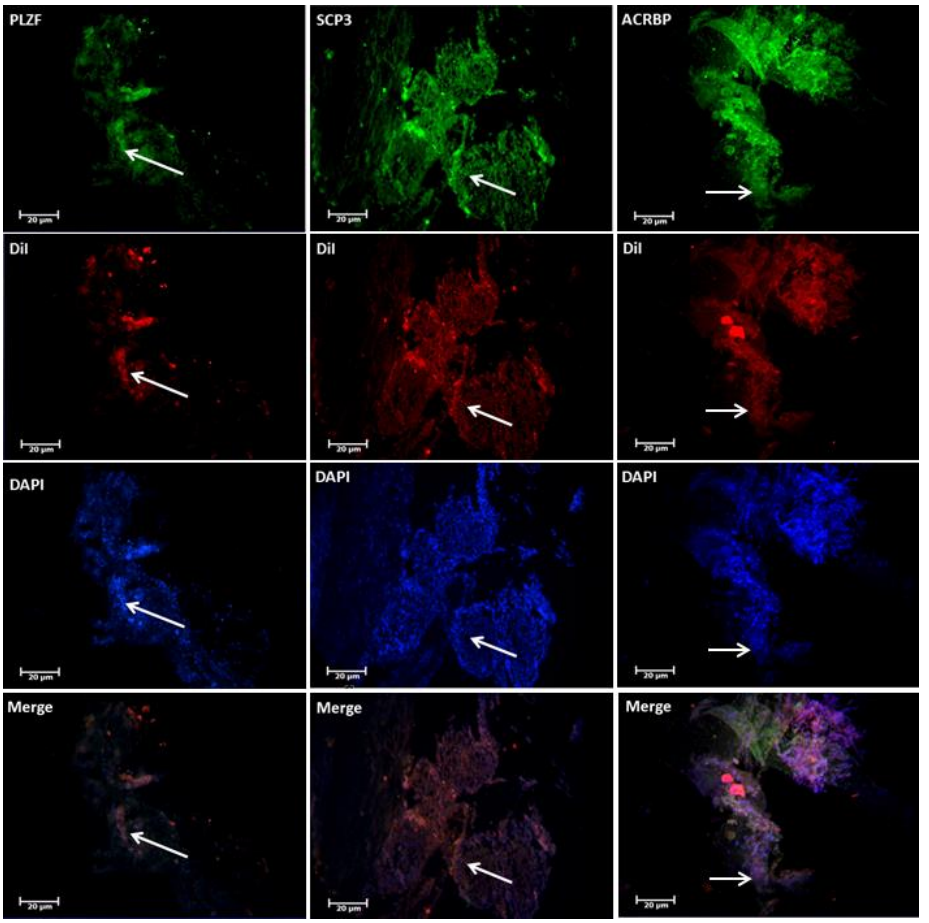

Figure 4. The immunohistochemistry and DiI tracing studies in host testes. Specific proteins of SCs (PLZF), spermatocytes (Tekt1) and spermatids or sperms (ACRBP) were expressed positively as same as DiI detection in host testes after 8 weeks of tissue culture in IVT group.

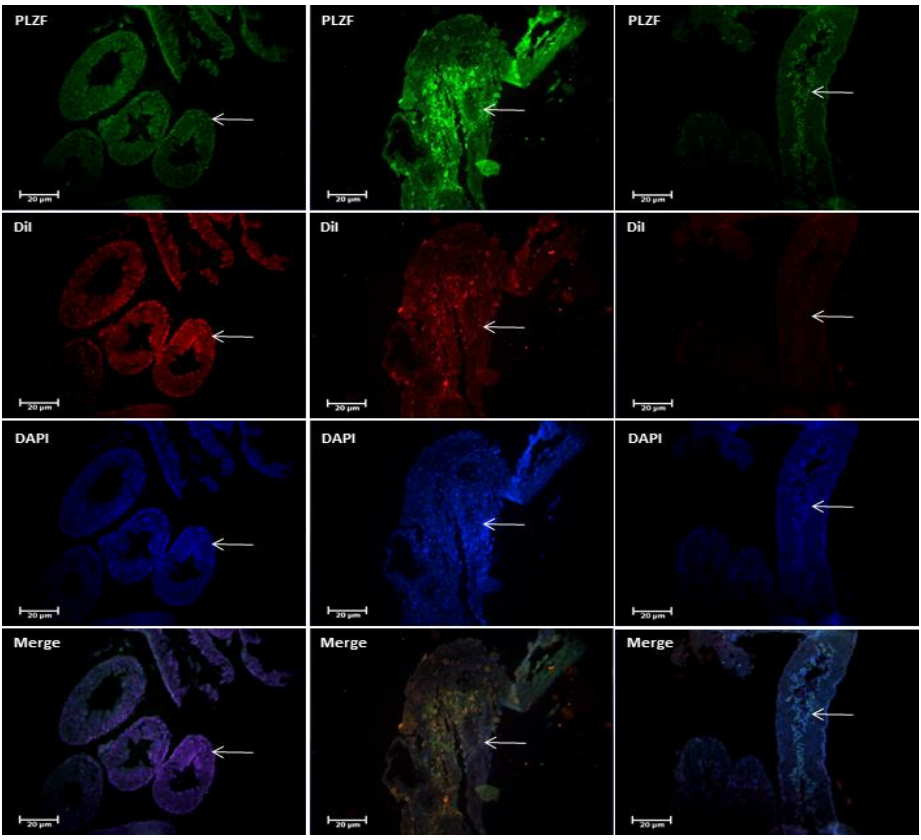

Figure 5. The immunohistochemistry and DiI tracing studies in host testes. Specific proteins of SCs (PLZF), spermatocytes (Tekt1) and spermatids or sperms (ACRBP) were expressed positively as same as DiI detection in host testes 8 weeks after transplantation in in vivo group.

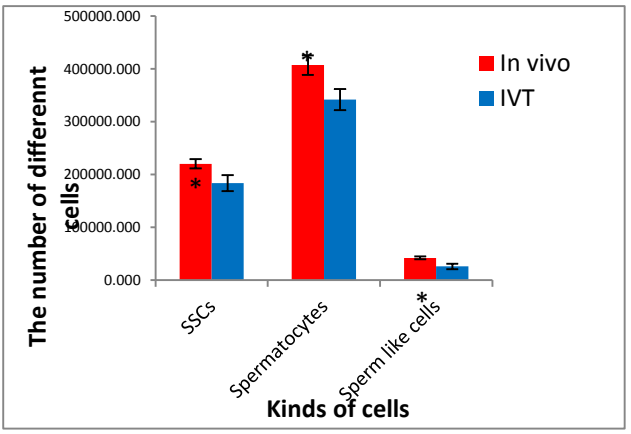

Chart. 1. Morphometric studies in host testes to determinate number of different types of cells of seminiferous tubule epithelium 8 weeks after transplantation in different groups.
*: Significant different with control group (P<0.05)

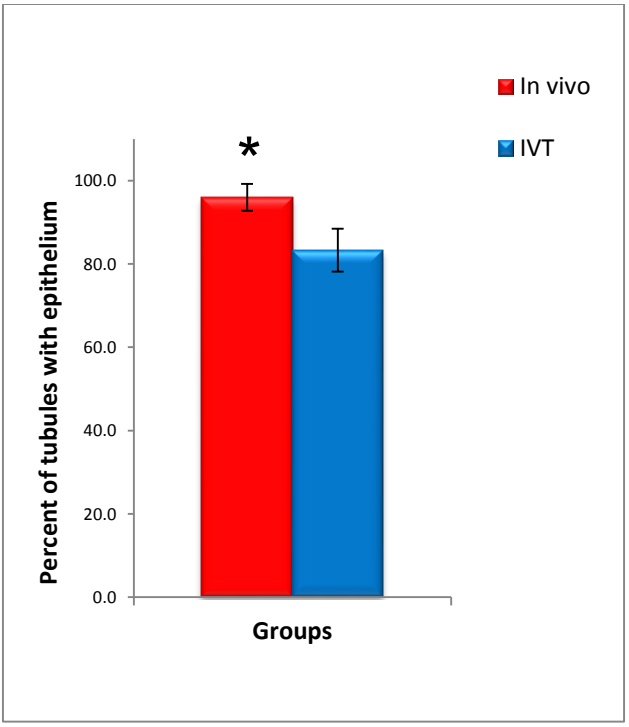

Chart 2. Percentage of seminiferous tubules with epithelium 8 weeks after transplantation in different groups.
*: Significant different with control group (P<0.05)

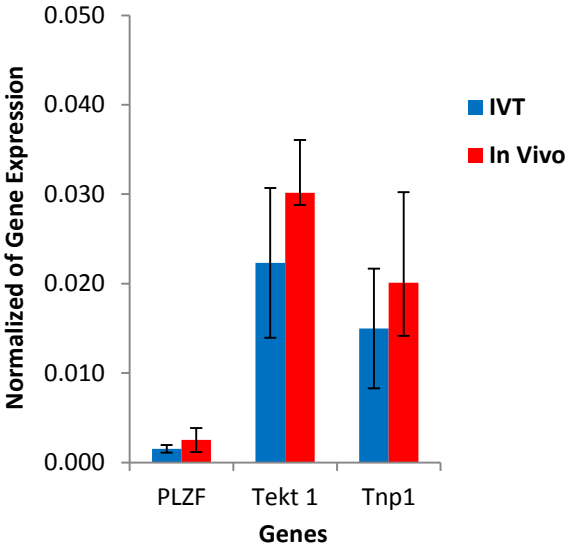

Chart 3. Expression of specific germ cells in the host testes 8 weeks after transplantation in different groups. There isn't significant difference.

Discussion

The transplantation of SSCs to azoospermia testis model was carried out by various researchers on various animal models. In the present study, we would like to compare the progress of spermatogenesis after the in vivo transplantation to IVT and then tissue culture of host testes. The results of histological studies and the detection of DiI in the host testis 8 weeks after transplantation has indicated the placement of transplanted SSCs on the basic membrane of the seminiferous tubules and the progression of spermatogenesis to the cells in the pulled in lumen centers in both in vitro culture and in vivo condition. The probability that these cells are located in the center of the seminiferous tubules with the nature of haploid or long spermatid sperm cells are confirmed by the results of Immunohistochemical and molecular studies in both groups.

SSC in vivo transplantation has now been reported in mice, rats, monkeys, goats, bulls, pigs, sheep, and dogs successfully [17]. It is important to demonstrate that transplanted SSCs can produce sperm in higher primate models that have the greatest relevance to human testis anatomy and physiology. Herman et al (2012) transplanted SSCs to rhesus host testes and theirs results indicated that SSCs from prepubertal or adult rhesus macaques could engraft chemotherapy-treated recipient testes and generate spermatogenesis, including the production of donor sperm that were

competent to fertilize rhesus oocytes resulting in preimplantation embryo development [18].

The findings of the IVT group are consistent with the results reported by Sato et al in 2013. Sato and colleagues were done in vitro transplantation of mouse SSCs into immature azoospermic mice testis that these transplanted cells sit down on a membrane of seminiferous tubules after 7 to 14 days. Their marking was to track Acrosin GFP in transplanted cells. They reported that after 40 to 50 days spermatids or sperm cells appeared [7]. However, the exact mechanism of the alignment of transplanted cells on the membrane has not yet been completely transparent [19]. Shinohara et al in 2008 suggested strongly that B1-integrin is involved in the first several weeks of SSC homing and colonization [20]. Mohaqiq et al in 2018 were shown that transplanted SSCs to host testes by the IVT could conclude to homing of these cells after 2 weeks of tissue culture of host testes, as same as the in vivo transplantation [2]. Sato et al in 2011 conducted another study in which germ cells were transplanted into seminiferous tubules in the in vitro condition. After placement on the membrane of the seminiferous tubules, these cells began to mitotic and then differentiated into higher-grade germ cells and eventually reached spermatid and haploid sperm cells, which were fertile, and by microinjection, fertile ability the ovum [21]. In the in vivo condition because of the presence of all characteristics of spermatogenesis both physically and nutritionally, it seems to take easy for spermatogenesis induction by SSCs transplantation [22]. However, the presence of interstitial tissue concludes Myoid cells, Leydig cells, etc are so necessary to support different mechanisms of spermatogenesis [23]. SSCs' fate is regulated by a complex interaction between growth factors secreted by the interstitial cells together with Sertoli cell and SSCs [24]. It is could be important that the production of Dihydrotestosterone is a major point in spermatogenesis progress by Sertoli cells functions [25]. We can preserve almost all of these conditions both physically and nutritionally in the in vitro culture systems as the results of previous studies.

It seems that the testis tissue culture system can induce the ability to support and resuscitate the spermatogenesis process by preserving the testicular paracrine environment including interstitial tissue and supportive cells in seminiferous tubules. The study results indicate that in vitro transplantation system and testicular tissue culture can support the progression of spermatogenesis to obtain haploid cells, as well as in vivo condition.

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Conflict of interest: We wish to confirm that there are no recognized conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Ethical statement: All stages of this research were based on the approval of the research ethics committee of Tarbiat Modares University with the registration ID IR.TMU.REC.1394.68. Informed consent was confirmed by the TMU.

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