Transplantation of Spermatogonial Stem Cells Suspension into Rete Testis of Azoospermia Mouse Model

Arefeh Jafarian[1], Niknam Lakpour[2], Mohammad Reza Sadeghi[3], Sheida Salehkhou[2], Mohammad Mehdi Akhondi[2]*

Purpose: The loss of spermatogonia following chemo-or radiotherapy leading to temporary or permanent infertility of the patient is a well known and unwanted side effect of many oncological therapies.

Materials and Methods: In this study, germ cells were isolated from 4 days old mouse testis cells. Busulfan treatment was used to the eliminate proliferating cells in the testis of recipient mice. The donor cells suspended in DMEM, were introduced into the rete testis of recipient mice via microinjection method. To distinguish the progeny of the transplanted donor stem cells from endogenous germ cells, BrdU-labeled cells were used. In addition, real time PCR was performed to determine expression levels of ngn3 and LIN28 (spermatogonia stem cells markers) before and after transplantation. Western blot analysis was further performed to detect an increase in - ngn3 expression after transplantation.

Results: Transplantations of stem cells into rete testis of the recipients was done. Our results clearly showed a significant increase in spermatozoa number in epididymal luman Spermatogonial stem cells (SSCs) did not show alkaline phosphatase activities while ngn3 and LIN28 were clearly expressed. Ngn3 and LIN28 expression were reduced after busulfan treatment compared to untreatmented mice. However, the expression of ngn3 and LIN28 increased after transplantation . BrdU-labeled testis cells were successfully transplanted into rete testis of recipient mice. These cells remained in rete testis of all recipient mice up to two months after transplantation.

Conclusion: The present study clearly confirme that a regeneration after cytotoxic treatment was based on morphological criteria. We demonstrated the increase in stem cell numbers during regeneration and after transplantation.. Transplantation of spermatogonial stem cells suspension by the injection of cells via the rete testis of recipient azoospermia model considerably enhances the efficiency of this procedure.

Keywords: rete testis; spermatogonial stem cells; transplantation.

INTRODUCTION

Germ cell transplantation technique has tremendous applications in a wide range of species. It was used to study spermatogonial stem cells (SSCs) biology, production of valuable transgenic animals and restore fertility in different animal species. Although recent progress in spermatogonial stem cell transplantation has been shown to restore fertility in sterile animals[(1)]. Some major problems are associated with stem cells enrichment, freezing methods and transplantation. To date, differentiation of SSCs into mature spermatozoa has not been demonstrated in vitro culture systems, and new strategies in germ cell transplantation has not been successful to produce enough mature sperm[(2)]. It has been reported in previous studies,SSCs culture before transplantation into recipient testes could increase the number of mature sperm production. SSCs transplantation is a promising strategy to preserve sterility, especially in pre pubertal boys treated with anti-cancer therapy[(3)]. Isolating and cryo freezing of SSCs in a boy with cancer is an important theoretical way to restore reproductive potential. This strategy, has been successful in animal models systems, however, it has not yet been applied in humans yet[(4)].Spermatogenesis is a complex biological process that takes place in seminiferous tubules where diploid spermatogonial divided by mitosis, meiosis and spermiogenesis process to produce mature sperm. The period required for spermatogenesis is different between various species. It takes approximately 74 days in human, 52 days in rat and 35 days in mice[(5)]. The process of spermatogenesis is divided into three different phases: spermatocytogenesis, meiosis and spermiogenesis. During the spermatocytogenesis, type A spermatogonia are divided mitotically to produce type B spermatogonia that rest on the basement membrane. In meiosic phase the primary spermatocytes undergo first meiotic division and become secondary spermatocytes and then the spermatid cells produced by second meiotic division. During spermiogenesis, the spherical and haploid spermatids develop into final product, spermatozoa. Spermatozoa are released into the lumen of the seminiferous tubule and carried toward the epididymis to achieving motility and become

[1]Immunology, Asthma and Allergy Research Institute (IAARI) , Tehran University of Medical Sciences, Tehran, Iran.
[2]Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.
[3]Monoclonal antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.
*Correspondence: Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, P.O. Box: 19615-1177, Tehran, Iran.
Tel.: +98 21 22432020. Fax: +98 21 22432021. E-mail: Akhondi@avicenna.ac.ir.
Received November 2016 & Accepted July 2017

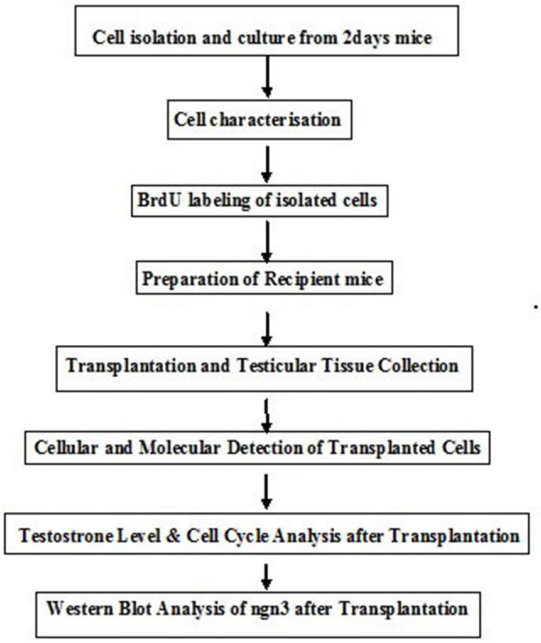

Figure 1. Spermatogonial stem cell culture and labeling
A: a) SSCs proliferation, b) SCs colonization
B: SSCs were positive with monoclonal anti-BrdU antibody in culture media. a) Single SSCs b) Negative control c) Colony of SSCs. Magnification: ×20

capable of fertilization. A relationship between Sertoli cells in somniferous epithelium has an important role in regulation of SSCs self renewal and spermatogonia optimal number[6]. Growth factors produced by the Sertoli cells have regulatory roles in induction or inhibition of self renewal and differentiation of germ cells[7,8]. One of the important advantages of germ cell transplantation is diagnosis of colons that is created from a single cell[9]. The most important cells that affected in radiation or chemotherapy are undifferentiated population of spermatogonia with mitotic activity. Recent studies have revealed that LIN28 and NEUROG3 are expressed by undifferentiated stem cells and progenitor spermatogonia, including Asingle, Apaired, and Aaligned 4–16[10,11]. LIN28 expression is associated with pluripotency and is not expressed in adult mouse testis[12]. Ngn3 is a class B bHLH transcription factor that have main function in the early steps of spermatogenesis[10]. Therefore, LIN28 and ngn3 could have ideal factors for transplantation assessment.Transplantation of male germ line stem cells opens another exciting strategy for fertility preservation[13]. Potential clinical applications of germ cell transplantation includes restoration of fertility in patients who undergo sterilizing treatments for malignancy or genetic defects in testicular somatic cells[14]. Efficient germ cells transplantation is dependent on the number of SSCs number, cell survival and hormonal regulation[15]. Spermatogonial cell transplantation is an efficient technique that can help to study of SSCs multiplication and renewal leading to to restoration of spermatogenesis[16]. In a previous study, we reportsuccessful transplantation of neonatal mouse donor testis cells into recipient seminiferous tubules[17]. While, in the present study, we used rete testis transplantation to increase the efficiency of SSCs transplantation. Therefore, the aim of this study is to evaluate the effect of rete testis transplantation on postnatal testicular germ cell proliferation following stem cell transplantation.

MATERIALS AND METHODS

Cell isolation and culture

C57BL/6 mice were purchased from the Pasteur Institute (Tehran, Iran) and were kept in conditions consistent with requirements of the local commission for ethical matters of animal experimentation. Testis were isolated from neonatal mice (2 to 4 days old afterdecapsulation under a dissection microscope, cells were suspended in DMEM Minimum containing; 0.5 mg/ml collagenase/dispase, 0.5 mg/ml trypsin and 0.05 mg/ml DNase supplemented with 14 mM $NaHCO_3$ (Sigma), non-essential amino acids, 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 µg/ml gentamycin (all from

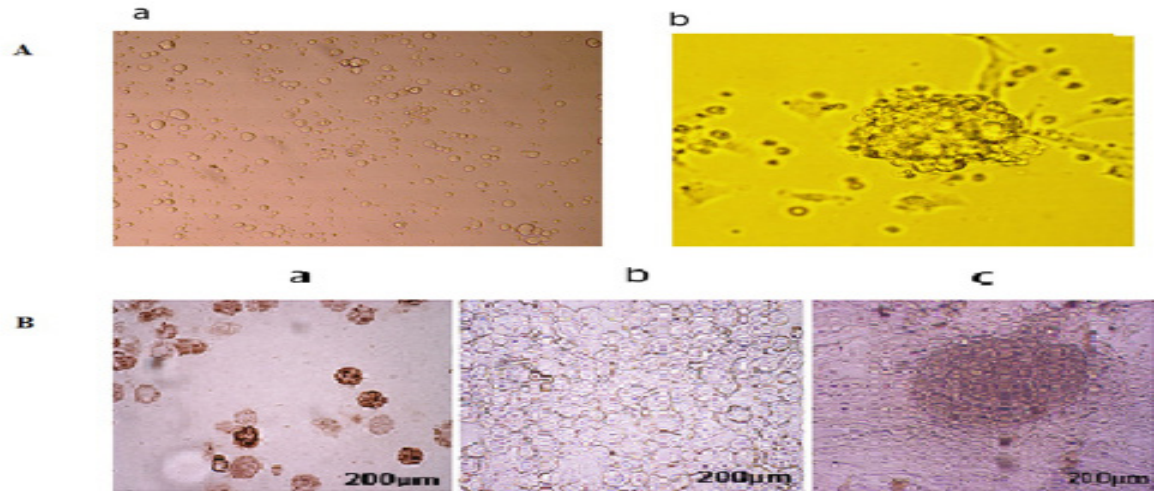

Figure 2. Alkaline phosphatase reactivity
a. The colony did not show alkaline phosphatase reactivity. Control groups including **b.** blood neutrophils cells; **c.** mouse intestine was alkaline phosphatase positive and **d.** Negative control. Magnification: × 20.

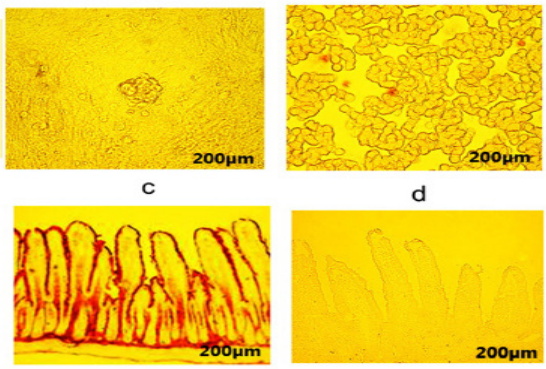

Figure 3. : Immunostaining of cytokeratin and ngn3
A) a) Cytokeratin was detected in the neonatal Sertoli cells b) Negative control.
B) B) ngn3 immunoreactivity was positive for the SSCs. Magnification: × 20
b) ngn3 immunoreactivity was positive for the SSCs. a) SSCs with HRP - conjugated anti mouse IgG as secondary antibody, b) SSCs with FITC - conjugated anti mouse IgG as secondary antibody, c) Colony of SSCs, d) Negative control. Magnification: × 20

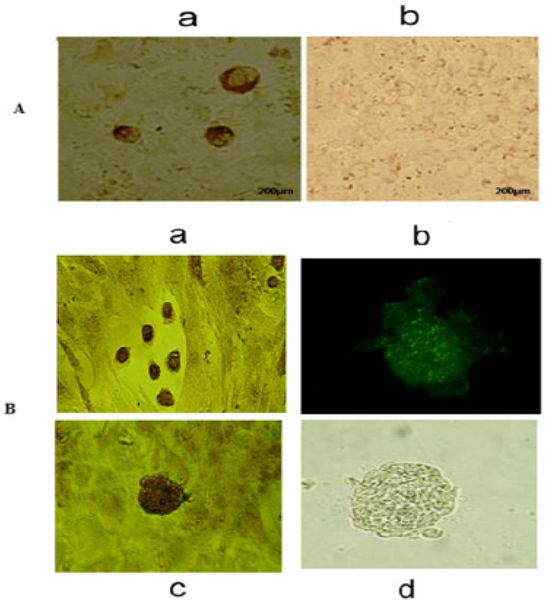

Figure 4. Testis histology and the regeneration of spermatogenesis after busulfan injection
Busulfan is a chemotherapeutic agent that induced azoospermia, testicular atrophy and depletion in spermatogenic cells a) Wild-type C57Bl/6 mouse testis (control), b) Busulfan treated testis at a dose of 30 mg/ kg 35 days after injection, c) Busulfan-treated testis at a dose of 30 mg/ kg 70 days after injection d) Busulfan- treated testis at a dose of 30 mg/ kg 100 days after injection, e) Capsular thickness in normal testis, f) Capsular thickness in azoospermia mouse testis. The results showed that testis capsular thickness was increased after busulfan treatment, g) Changes in testis size after injection of busulfan. Busulfan decreased the testis size and depleted spermatogenesis. a) Normal testis, b) Neonatal mouse testis, c) Busulfan treated mouse testis Magnification: × 20

Invitrogen, Carlsbad, Calif, USA). They were incubated for 45 min at 37 °C with shaking and gentle pipetting. Then the supernatant containing the Leydig cells was removed and the cells were incubated in DMEM medium containing collagenase (1.5 mg/ml), hyaluronidase (1.5 mg/ml), trypsin (0.5 mg/ml), and DNase (1 µg/ml) for 20-30 min at 37°C and 5% CO2. Subsequently, the cells were cultured at 32 °C in 5% CO_2 at a concentration of 10×106 cells/dish. After trypsin digestion the cells washed and seeded onto new dishes with DMEM medium for transplantation into recipient testes. Total cell number and viability were determined before transplantation[18]. The schematic flowchart of methods is shown in **Figure 1**.

Alkaline phosphatase (AP) activity

After 3 days cultured cells were fixed in 4% paraformaldehyde at room temperature for 1 min and washed twice with PBS then stained with Alkaline phosphatase substrate solution (Sigma) for 30 min at 37°C. Then AP-activity was detected colorimetrically by visual analysis of the stained cells red bright color indicating expression of alkaline phosphatase against a yellow background. Tissue cryosections was used as a positive control[19].

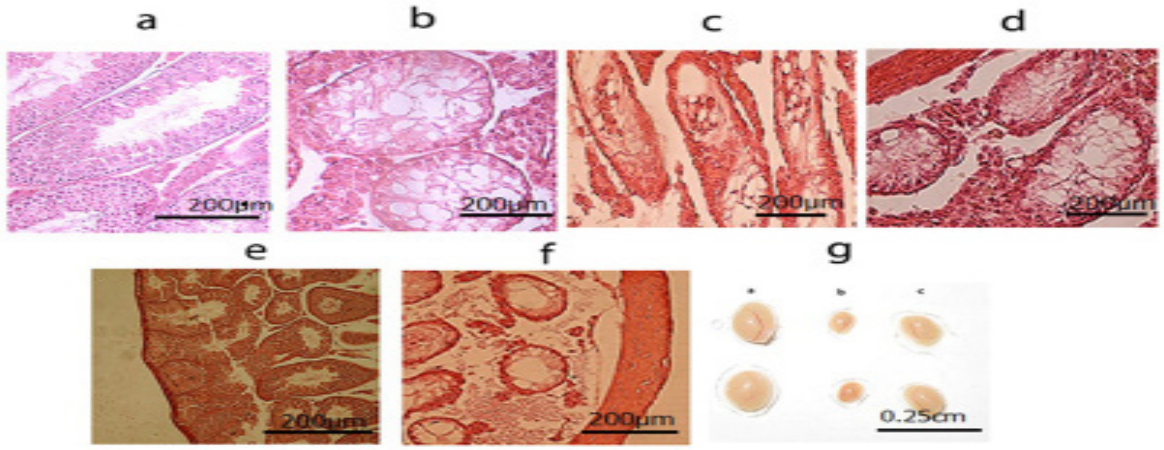

Figure 5. BrdU staining following spermatogonial stem cell transplantation
(a) Spermatogonial stem cell detection in transplanted testis cryosection staining. (b) Negative control of BrdU Staining in testis tissue. Magnification: × 40

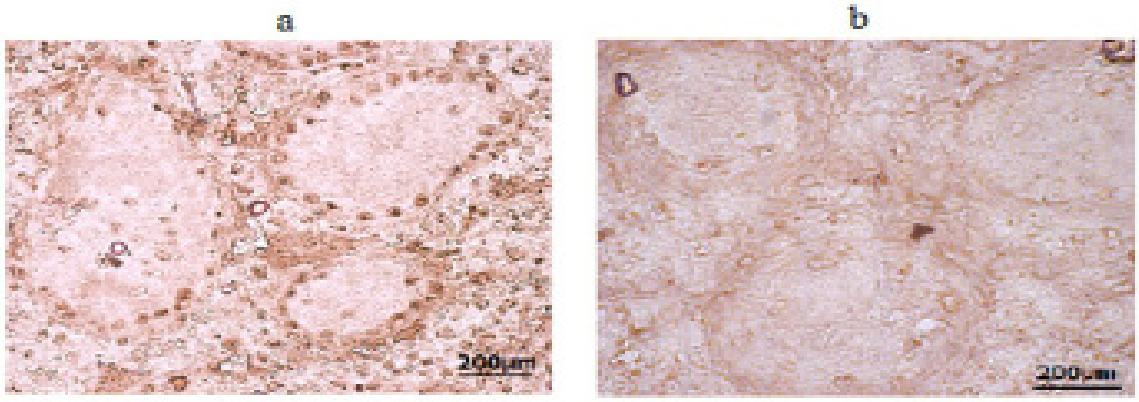

Figure 6. qRT-PCR analysis of ngn3 and LIN28 in azoospermia mouse SSCs
A) The ngn3 was exclusively expressed in testis cells of adult mouse, 3-6 days old mice testis but decreased busulfan treated mouse testis after transplantation its expression increased. P value < 0.05
B) LIN28 expression decreased in adult busulfan treated mouse testis. Results revealed that LIN28 expression increased after transplantation. . *P* value < 0.05

Immunohistochemical localization of Cytokeratin

Sertoli cells were studied using an antibody to Cytokeratin-18 (CK-18). Isolated cells were centrifuged at 30 × g for 5 min in a Cytospincentrifuge (Shandon, Cheshire, UK) and fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20 minutes. Then the cells washed 3 times with PBS containing 0.5 % Triton X- 100 and 10% rabbit serum. Unspecific site was blocked with 10% sheep serum in PBS for 30 min at room temperature. Subsequently, the cells were incubated at 37 °C for 1 hr with 1:100 dilution of mouse primary monoclonal anticytokeratin Pan (Boeringer-Mannheim, Germany) antibody. The cells were washed and incubated with FITC-coupled (1:50) anti mouse IgG as secondary antibody (Sigma) for 45 min.. The slides were visualized under a fluorescence microscope (Nikon.US). All experiments were repeated at least two times[20].

Immunocytochemical staining of ngn3

The cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20 minutes and washed 3 times with PBS containing 0.5 % Triton X- 100 and 10% rabbit serum. Unspecific site was blocked with 10% sheep serum in PBS for 30 min at room temperature. Subsequently, the cells were incubated at 37 °C for 1 hr with 1:100 dilution of mouse primary anti ngn3 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the cells were washed in PBS and incubated with 1:50 dilution of Horse Radish Peroxidase (HRP) - conjugated anti mouse IgG as a secondary antibody (Sigma) for 45 min. After washing with TBS/BSA the color was developed by the addition of 3, 3'-Diaminobenzidine (DAB; Sigma) for 8-10 min and, the slides were assessed with an optical microscope[20].

Incorporation of 5-Bromo-2 deoxyuridine (BrdU) in spermatogonial stem cells

To trace the transplanted cells, the cells were incorporated with 5-bromo-2′-deoxyuridine (BrdU, Sigma) before transplantation. After reaching approximatly 70% confluency, BrdU incorporation was performed by adding 0.1 mM BrdU to the culture medium for 24 hr. Then, the BrdU incorporated cells were centrifuged at 500g for 4 min. After fixation in ice-cold acetone, the cells were washed in PBS containing 1% BSA, 0.05% Tween 20, and 0.1% sodium azide. DNA denaturation was done by 2N HCl for 30 minutes at 37 °C. Monoclonal anti BrdU antibody (Sigma1:500) was added to

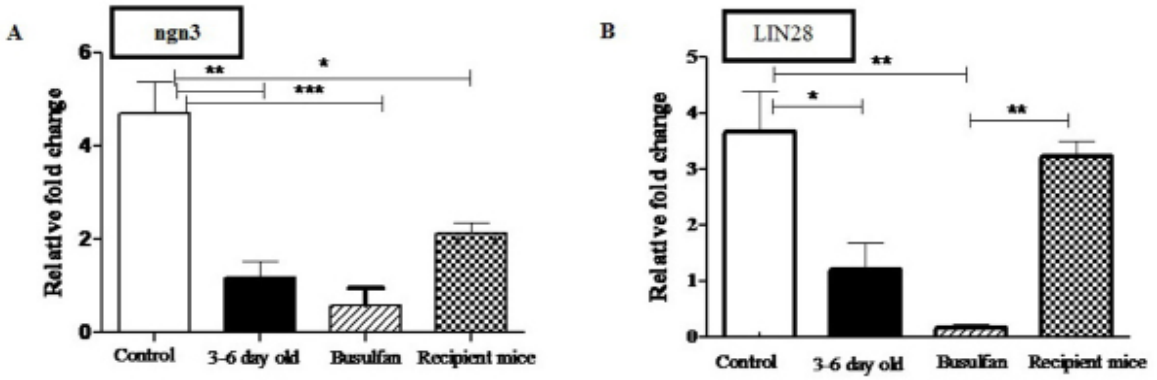

Figure 7. Serum level of testosterone and germ cells numbers
Two months after transplantation A) Testosterone level and B) Number of haploid cells percent were shown to have an increase in transplanted mice in compared to the control group ($p < 0.0005$)

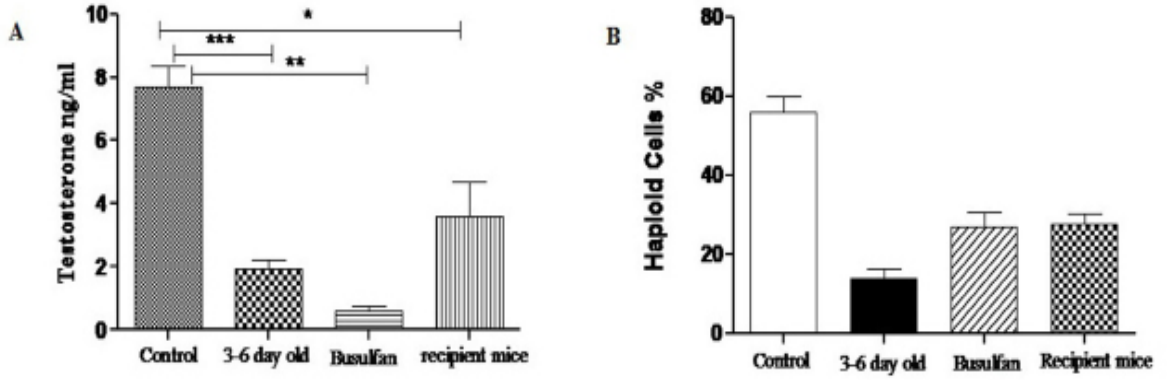

Figure 8. Serum level of testerone and germ cells numbers.

the cells for 2 hr at 37 °C. Subsequently, the cells were incubated for 45 min with Horse Radish Peroxidase (HRP)-conjugated anti mouse IgG (1:50) as a secondary antibody. Then, 10% DAB solution was applied for 10 min. The BrdU positive cells were observed under inverted phase contrast microscope.

Preparation of Recipient mice

Busulfan was used to deplete endogenous germ cells and degeneration of spermatogenesis in order to prepare recipients. Adult male C57Bl/6 mice (6 weeks of age) were kept at stable temperature (22 °C) and light–dark cycle of 12 h light/12 h darkness with free access to food and water. Busulfan (Sigma Chemical, Dorset, UK) was dissolved in dimethyl sulphoxide (DMSO; Sigma), and an equal volume of sterile distilled water was added to provide final concentrations of 30 mg/ kg. Adult mice received a single intraperitoneal injection of busulfan[15].

Transplantation and Testicular Tissue Collection

The donor spermatogonia and Sertoli cells in suspension were suspended in a volume of approximately 15 µl of DMEM/ FCS (fetal calf serum) and transplanted into rete testis of recipient mice with a microinjection needle. The recipient mice were anesthetized for transplantation. Transplantation was carried out a week after culturing. For testicular injections, the cells (1x 106) were maintained on ice and then microinjected into the rete testes using glass needle in one of the testes of each recipient mice; and another testis was used as an internal control. The testes of the recipient mice were collected and fixed in 10% neutral buffered formalin (Merck, Darmstadt, Germany) or kept in −80 °C for paraffin sections and spermatogenic markers were detected immediately after the injection at 4 and 8 weeks. For evaluation of spermatogonial stem cell colonization, recipient mice testes were analyzed with BrdU stainingThe transplanted testis sections with 5 µm thickness were immunostained with a primary anti-BrdU in order to visualize the donor-derived spermatogenesis.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from fresh testis tissues using Trizol (Invitrogen) according to the manufacturer's instructions. The first strand cDNA was synthesized with 50 ng of total RNA by random hexamer priming using high capacity cDNA synthesis kit (iNtRON, Korea) at 42°c for 60 min and at 70°c for 5 min. Quantitative real-time PCR was performed with a SYBR-Green kit (Takara, Korea) according to the manufacturer's instructions and using the ABI Light Cycler (ABI step one) in a total reaction volume of 10µl to analyze the expression of ngn3 and LIN28. All reactions were performed in triplicate and results were normalized to GAPDH (internal control) to correct RNA input in re-

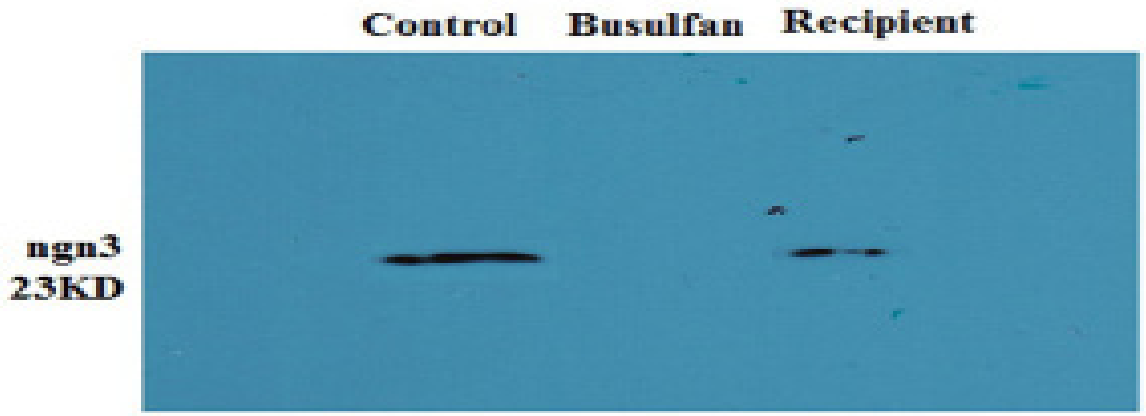

Figure 9. ngn3 detection by western blot in mouse testis
Western blot analysis detected presence of ngn3 protein in the normal testis. Busulfan treatment caused complete depletion of germ cells, and after transplantation ngn3 expression was showed in recipient mouse testis.

actions. The following primers were used for RT-PCR: Lin28 primers: AGACCAACCATTTGGAGTGC and AATCGAAACCCGTGAGACAC;ngn3primers: GTTGGTGAGCCCCTGGAGACCATAT and CTGGCCCCTGGCCCCTGGGCAC ;GAPDHprimers: AAGGTCATCCCAGAGCTGAAand CTGCTTCACCACCTTCTTGA.

Determination of Testosterone levels and Germ cells Number

For measurement of testosterone, the mice were anaesthetized with 0.64 mg/ kg xylazin (Alfasan, Woerden, and the Netherlands) and 20 mg/ kg ketamine (Alfasan). Serum testosterone level was measured by a chemiluminescence analyser (Diasorin kit; Italy). The blood of animal was collected by cardiac puncture. The serum after separation was stored at 20 °C for testosterone level detection. For DNA flow cytometry, freshly harvested testicular tissue was separated and placed in phosphate-buffered saline (PBS). Enzymatic digestion of testicular tubules was performed after testicular tissue dissection according to the technique of Dym et al. with minor modifications . To remove residual cells, tissue fragments were washed twice with phosphate buffer. Then, 1 ml of cold 70% ethanol was added drop by drop to the obtained sediment. After centrifugation (10 min in 500 g), the supernatant was discarded. Then, 0.5 ml of nucleic acid staining solution consisting of 8.5 ml of staining buffer (0.1% BSA in PBS), 0.1% RNase (Sigma) and 0.5 ml of stock solution of propidium iodide (1 ng/ ml; Sigma) was added to the pellet. DNA histograms were obtained by a flow cytometer (Becton-Dickinson, FAC scan, San Jose, CA, USA), and analysis was performed by CELL QUEST programs (Becton-Dickinson) (15).

SDS-PAGE and Western Blotting

Proteins were extracted from the testis of 6 weeks old mice (control) and busulfan-treated mice using Radioimmunoprecipitation assay (RIPA) buffer containing 10mM Tris-HCl (PH = 8.0), 1% NP-40, 10% Glycerol, 0.1% SDS,1mM EDTA and 100mM Nacl with protease inhibitor cocktail (Roche Diagnostic, GmbH, Germany). 50 µg of total protein was heated to 95°C for 5 min and separated by 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and blotted on to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences). Protein binding sites were blocked for 2 hrs in 3% nonfat skim milk in Tris-buffered solution with 0.1% Tween 20 (TBST) at room temperature (RT). The membrane was incubated overnight at 4 °C in 1 % BSA solution containing 1:300 dilution of primary antibody (ngn3 monoclonal antibody, Santa Cruz) the membrane was diluted with 1:10,000 HRP conjugated rabbit anti mouse immunoglobulins (Sigma) 1hr at room temperature after 3 times of washing with TBST, bands were visualized using enhanced chemiluminescence (ECL) reagent (Ariyatous Biotech, Iran) according to the manufacturer's instructions and were compared with □-actin.

Statistical analysis

The values are presented as the mean ± SEM. Results were analyzed by one-way ANOVA and Bonferroni's post-hoc test was used for comparison of experimental groups with control by Graph pad Prism5 software. P-value less than .05 was considered statistically significant.

RESULTS

Cell culture and labeling

Approximately before 1 week several small colonies were observed on top of the monolayer of testicular cells (**Figure 2a**). Isolated SSCs was incubated in medium with 0.1 mM BrdU and the BrdU labeled cells were used for transplantation. To in vitro detection of these cells after rete testis transplantation, monoclonal anti-BrdU antibody was used in culture medium and BrdU positive cells were observed by optical micscope

(**Figure 2b**).

Alkaline phosphatase (AP) activity

Cultured spermatogonial stem cells did not show any alkaline phosphatase activity. Althogh, Alkaline phosphatase is highly expressed in the mouse intestine, as well as blood noutrophil cells that served as a positive control(**Figure 3)**.

Immunostaining of cytokeratin and ngn3

Immunocytochemical evaluation using an antibody against cytokeratin showed cytoplasmic localization of CK-18 in Sertoli cells (**Figure 4a**). Figure 4b clearly showes a positive expression of ngn3 in SSCs compared to control.

Recipient mice

Four weeks after the injection of mice with busulfan, most of the endogenous germ cells were removed (**Figure 5a**). Histological analysis showed that 70 and 100 days after injection of 30 mg/ kg busulfan, the regeneration of spermatogenesis occurred in a few tubules, while it was not effective to return fertility in azoospermia mice (p = 0.005) (**Figure 5b,c**). On the other hand, evaluation by optical microscopy demonstated increase in capsular thickness after busulfan treatment compared to the control group (**Figure 5d**).

Donor cells in recipient seminiferous tubules

Two months after transplantation of cells into rete testis, most of the donor cells were observed in tubule cross-sections of recipient mice as detected by immonohistochemistry for BrdU (**Figure 6a**). No BrdU positive cells were found in the non-transplanted group (**Figure 6b**).

Expression of spermatogenic molecular markers in recipient testicular tissue

The known molecular markers of spermatogonial stem cells and spermatogonia ngn3 and LIN28 were detected in adult (control group), 3-6 days old, busulfan treated and recipient mice testes as determined by q RT-PCR. Expressions of ngn3 and LIN28 at relatively high levels were detected 8 weeks after transplantation in recipient mice (P < .05) (**Figure 7**).

Testosterone concentration and cycle analysis

Two months after transplantation, testosterone level and number of haploid cells percentage were shown to have an increase in transplanted mice compared to control group (P = .0005) (Figure 8a, b).

Detection of ngn3 protein level by western blot in spermatogonial stem cells

Western blot analysis detected the presence of ngn3 protein in the testis. The ngn3 antibody recognized a single protein band of ~ 23 kDa in normal mice. Western blot results indicated no detectable ngn3 protein in busulfan treated testis. As a result, busulfan treatment

caused a complete depletion of germ cells, and thus these animals were used as recipients for germ cell transplantation (**Figure 9**).

DISCUSSION

The germ cell transplantation technique has provided a new treatment approach in restoration of fertility in oncological patient. In this propose, azoospermic mice are a good model for development of this new technology. In the past, there was not an appropriate in vitro culture condition to support proliferation and differentiation of SSCs for a long time. Although, a complete in vitro spermatogenesis has not been obtained in any species[21]. In the mature testis, 2-3 stem cells exist in 104 testicular cells. Therefore, in this study we used testes from immature mice to provide a large number of undifferentiated SSCs[22]. Stem cell expansion following busulfan treatment and before transplantation had effective role in cell population. Before autologous transplantation and treatment of infertility, expansion of SSCs is necessary. According to recent reports, c-kit is express in differentiating spermatogonia (Aa116 and possibly Aa18) and undifferentiated SSCs are c-kit negative[10,23,24]. While, ngn3 and LIN28 are expressed by spermatogonial stem cells and early steps of spermatogenesis[11]. So, we used ngn3 and LIN28 to characterization of cultured SSCs. In the present study, germ cell transplantation from rete testis of azoospermia mouse model copuld lead to an increase in efficiency of transplantation. The results of busulfan injection to deplete endogenous germ cells confirmed the regeneration is based on stem cell expansion after cytotoxic treatment. After busulfan injection and destraction of primitive germ cells that account for less than 1% of the total number of testis cells[25,26], nearly all of the differentiated progenitor cells remained and continued their differentiation with normal kinetics. However, due to the absence of self-renewal activity in SSCs they gradually got mature and disappeared by 35 days[27,28]. Therefore, as the stem cell numbers continued to recover, the number of differentiated germ cells decreased, and the ratio of stem cells to differentiated germ cells markedly changed during regeneration. Our results apparently revealed the number of stem cells decreased and reached the lowest value four weeks after the injection of 30 mg/kg busulfan. However, stem cells then started to increase and histological analysis showed the regeneration of spermatogenesis in a few tubules between 70 and 100 days after injection of 30 mg/ kg busulfan while, stem cell regeneration was not effective. Because it has been reported that Busulfan is a potent agent that can destroy Sertoli cells in testis environment[29]. So, abnormal testis environment may decrease spermatogenesis efficacy. In the present study, we used neonatal or prepubertal donor testis cells with most of undifferentiated cells in suspension. So, 25-30 µl of fresh cell suspension (~ 2/5×106 cells/ml) was slowly injected into the rete testis if azoospermicmouse. Then, SSCs migration into the niche or basement membrane of seminiferouse tubules and donor derived spermatogenesis was detected two month after transplantation. Previouse studies showed spermatogonial stem cells numbers in donor cell suspension is an important factor in colonization. An increase in SSCs population rather than differentiated cells could increase spermatogenesis efficacy. Indeed, cell cycle stages had a main role in regulating SSC migration to the niche[30]. In this study, further cell cycle analysis detected the presense of the most cells in the G2/M phase after transplantation that showed an success in homing of transplanted cells and progression into differentiation cycle. Although, the mechanism that promoteSSCs homing, replicattion and differentiation into mature spermatoza is complicated[31]. On the other hand, busulfan treatment induced stem cell degradation and testis tissue became approximately empty of SSCs. In this regard, two months after transplantation, the presence of transplanted cells was investigated by examination of ngn3 and LIN28 expression. Increased expressions of ngn3 and LIN28 could confirm the existence of new cells in testis of azoospermia mouse model. Based on recent finding, ngn3 and LIN28 were specifically expressed in the spermatogonia and busulfan could induce morphological damage to undifferentiated SSCs and sperm production. In our study, transplanted SSCs into rete testis of azoospermia mice, could to lead the cell suspension in near all of the tubules and that revealed by anti-BrdU staining. It was better to use GFP-donor mice to better distinguish testis resettlement, but it was limitations of our study. Two months after transplantation, an increase in ngn3 and LIN28 expressions revealed the success in SSCs homing and beginning of donor derived spermatogenesis in the recipient seminiferous tubules. We could success to evaluate SSCs transplantation in rete testis of azoospermia mouse model by cellular and molecular analysis. While, we need more time to check functional sperm in the ejaculate.

CONCLUSIONS

Germ cell transplantation was initially developed in rodents and had great potential and promising initial results. Number of important factors including isolation of donor cells, delivery to recipient testes and recipient animal preparation related to the success rate of the technique and making this procedure as a viable option for application. Clinical applications include preservation of fertility in patients undergoing potentially sterilizing treatments for cancer therapy and rare animals. In the practice the introduction of genetic modifications into the germline of domestic animals is most applications of SSCs transplantation. Rete testis transplantation of germ cells is newer approach that enhances our understanding of testis function, preserve fertility, study and manipulates spermatogenesis in a variety of mammalian specie

REFERENCES

1. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. Nature medicine. 2000;6:29-34.
2. Brinster CJ, Ryu B-Y, Avarbock MR, Karagenc L, Brinster RL, Orwig KE. Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biology of Reproduction. 2003;69:412-20.
3. Revel A, Revel-Vilk S. Pediatric fertility preservation: is it time to offer testicular tissue cryopreservation? Molecular and cellular endocrinology. 2008;282:143-9.
4. Wu X, Goodyear SM, Abramowitz LK, et

al. Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Human reproduction. 2012des077.

5. Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. The Anatomical Record. 1971;169:533-57.
6. Nagano M, Avarbock MR, Brinster RL. Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biology of Reproduction. 1999;60:1429-36.
7. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proceedings of the National Academy of Sciences. 1994;91:11298-302.
8. Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. Proceedings of the National Academy of Sciences. 1994;91:11303-7.
9. Lodi D, Iannitti T, Palmieri B. Stem cells in clinical practice: applications and warnings. J Exp Clin Cancer Res. 2011;30:1-20.
10. Yoshida S, Takakura A, Ohbo K, et al. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol. 2004;269:447-58.
11. Zheng K, Wu X, Kaestner KH, Wang PJ. The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol. 2009;9:38.
12. Yang DH, Moss EG. Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. Gene Expr Patterns. 2003;3:719-26.
13. Schlatt S, Ehmcke J, Jahnukainen K. Testicular stem cells for fertility preservation: preclinical studies on male germ cell transplantation and testicular grafting. Pediatric blood & cancer. 2009;53:274-80.
14. Dobrinski I. Germ cell transplantation. Paper presented at: Seminars in reproductive medicine, 2005.
15. Jafarian A, Sadeghi M, Pejhan N, Salehkhou S, Lakpour N, Akhondi M. Regeneration of spermatogenesis in a mouse model of azoospermia by follicle-stimulating hormone and oestradiol. Andrologia. 2014;46:1098-106.
16. Brinster RL. Germline stem cell transplantation and transgenesis. Science. 2002;296:2174-6.
17. Akhondi MM, Najar RA, Jeddi-Tehrani M, et al. The effect of human chorionic gonadotropin treatment on recipient mouse germ cell proliferation following spermatogonial stem cell transplantation of neonatal donor mice. Avicenna journal of medical biotechnology. 2010;2:23.
18. Schlatt S, Rosiepen G, Weinbauer G, Rolf C, Brook P, Nieschlag E. Germ cell transfer into rat, bovine, monkey and human testes. Human reproduction. 1999;14:144-50.
19. Anderson WA. Cytochemistry of sea urchin gametes: III. Acid and alkaline phosphatase activity of spermatozoa and fertilization. Journal of ultrastructure research. 1968;25:1-14.
20. Heidari B, Gifani M, Shirazi A, et al. Enrichment of undifferentiated type A spermatogonia from goat testis using discontinuous percoll density gradient and differential plating. Avicenna journal of medical biotechnology. 2014;6:94.
21. Guerin J. [Testicular tissue cryoconservation for prepubertal boy: indications and feasibility]. Gynecologie, obstetrique & fertilite. 2005;33:804-8.
22. Bellve AR, Cavicchia J, Millette CF, O'brien DA, Bhatnagar Y, Dym M. Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization. The Journal of cell biology. 1977;74:68-85.
23. Oatley JM, Kaucher AV, Avarbock MR, Brinster RL. Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. Biol Reprod. 2010;83:427-33.
24. Zhang M, Zhou H, Zheng C, et al. The roles of testicular c-kit positive cells in de novo morphogenesis of testis. Sci Rep. 2014;4:5936.
25. Kramer MF, de Rooij DG. The effect of three alkylating agents on the seminiferous epithelium of rodents. II. Cytotoxic effect. Virchows Arch B Cell Pathol. 1970;4:276-82.
26. Bellve AR. Purification, culture, and fractionation of spermatogenic cells. Methods Enzymol. 1993;225:84-113.
27. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl. 2000;21:776-98.
28. Meistrich ML. Effects of chemotherapy and radiotherapy on spermatogenesis. Eur Urol. 1993;23:136-41; discussion 42.
29. Hemsworth BN, Jackson H. Effect of Busulphan on the developing gonad of the male rat. J Reprod Fertil. 1963;5:187-94.
30. Ishii K, Kanatsu-Shinohara M, Shinohara T. Cell-cycle-dependent colonization of mouse spermatogonial stem cells after transplantation into seminiferous tubules. J Reprod Dev. 2014;60:37-46.
31. Cheung HH, Rennert OM. Generation of fertile sperm in a culture dish: clinical implications. Asian J Androl. 2011;13:618-9.