

# The Effect of Human Chorionic Gonadotropin Treatment on Recipient Mouse Germ Cell Proliferation Following Spermatogonial Stem Cell Transplantation of Neonatal Donor Mice

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## Abstract

Spermatogonia are the male germ line stem cells whose life long expansion is needed for permanent production of spermatozoa. The present study was designed to examine the effect of hCG treatment on germ cell proliferation following stem cell transplantation in mice. Spermatogonial stem cells were isolated from neonatal mice testes and characterized by alkaline phosphatase, immunoreactivity and morphological analysis. hCG was injected into normal and cell transplanted mice. We then evaluated the testosterone levels and cell number in normal mice. After that, *cyclin B1* gene expression was investigated in transplanted mice. Different doses of busulfan were injected to investigate the effects of chemotherapy on morphological criteria and preparation of recipient mice for transplantation. In this report we show proliferative potential of spermatogonial stem cells after cytotoxic treatment, transplantation efficiency by semi-quantitative RT-PCR, and hCG effect on stem cell regeneration in normal mice and following cell transplantation. The results indicate that spermatogonial stem cells can proliferate after transplantation, and the efficiency of their transplantation depends on hormonal treatment. Therefore, hormonal treatment after stem cell transplantation will be a powerful avenue for increasing the efficiency of transplantation and fertility restoration.

*Avicenna J Med Biotech* 2010; 2(1): 23-35

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Received: 30 Aug 2009

Accepted: 4 Nov 2009

**Keywords:** Busulfan, hCG, Cell proliferation, Transplantation

## Introduction

Proliferation and self-renewal of spermatogonial stem cells is based on the presence of hormonal support and growth factors<sup>(1)</sup>. Hormonal support is very important for proliferation of germ cells<sup>(2)</sup> and can be used for increase in transplantation efficiency. It is postulated that germ cell development is controlled by the hypothalamic-pituitary-gonadal axis<sup>(3)</sup> and established that spermatogenesis

is gonadotropin-dependent and optimal function of the testis is supported by FSH and by intratesticular androgens induced by luteinizing hormone<sup>(4)</sup>. Human chorionic gonadotropin (hCG) has more than 90% similarity with Luteinizing Hormone (LH) in structure and function<sup>(5)</sup> and therefore can be used alternatively. Androgen production is induced by LH and promotes germ cell

proliferation and maturation. Hypophysectomy or neutralization of circulating gonadotropins increases degeneration of spermatogenic cells<sup>(6-8)</sup>.

Spermatogonial cell transplantation is an efficient technique that can help infertility treatment and leads to restoration of spermatogenesis<sup>(9)</sup>. It may be proposed to preserve a fraction of the testicular stem cell of the patient that awaits cancer chemo or radiotherapy for use as auto-transplant after treatment<sup>(9)</sup>. This may be especially effective for prepubertal boys in whom spermatogenesis has not started and thus are lacking sperm.

Combining germ cell transplantation with culture and germ cell population enrichment of spermatogonia and hormonal support opens new pathways for conservation of livestock and plays important roles in medical sciences and reproduction. Events that cause an excessive loss of germ cells such as exposure to high doses of irradiation stimulate their proliferation<sup>(10, 11)</sup>. Self-renewal and differentiation of the testis stem cells is dependent on their close association with Sertoli cells in the seminiferous epithelium<sup>(12)</sup>. These processes are mediated by growth factors produced by the Sertoli cells, which induce or inhibit self-renewal, differentiation and further development of all germ cells<sup>(13, 14)</sup>.

Hormonal environment following transplantation is an essential factor for effective proliferation of transplanted stem cells and absence of such hormonal support may lead to failure in stem cell function and transplantation efficacy. Previously the effects of hCG injection on postpubertal germ cell maturation and androgen production in rat testis was evaluated<sup>(15)</sup>.

However, to our knowledge, the effects of hCG treatment on germ cell status and their proliferation before and after spermatogonial stem cell transplantation has not been studied. Therefore, the aim of this study is to evaluate the effect of different doses of hCG on postnatal testicular germ cell proliferation in normal and following stem cell transplantation.

## Materials and Methods

### *Isolation and culture of mouse type A spermatogonia*

Sequential enzymatic digestion of testicular tubules was performed essentially according to the method of Dym et al<sup>(16)</sup> with minor modifications. Briefly, testes of ten C57BL/6 3 to 6 days old mice were isolated, placed into a Petri dish, and covered with sterile Phosphate Buffered Saline (PBS).

Testes were decapsulated under a dissection microscope and were suspended in Dulbecco's Minimum Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) containing 0.5 mg/ml collagenase/dispase, 0.5 mg/ml trypsin and 0.08 mg/ml DNase (all from Sigma) supplemented with 14 mM NaHCO<sub>3</sub> (Sigma), single-strength non-essential amino acids, 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 µg/ml gentamycin (all from Invitrogen, Carlsbad, Calif, USA). They were incubated for 30 min at 37 °C on a shaker with occasional gentle pipetting to dissociate the interstitial Leydig cells from the seminiferous tubes. After this incubation step, the tubules were allowed to settle down and the supernatant containing the Leydig cells was pipetted out. The cells were then 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) (all from Sigma) for 20-30 min using the conditions described above.

The dispersed cells were then washed twice with DMEM medium and filtered through 70 µm nylon mesh (BD Falcon, Becton-Dickinson, USA). Isolated cells were cultured at 32 °C in 5% CO<sub>2</sub> in air on 5 cm plastic dishes at a concentration of 10×10<sup>6</sup> cells/dish. The cells were expanded by several passages of selected cultures by digesting with trypsin and were seeded onto new dishes. They were then harvested after trypsin digestion, washed in DMEM medium by centrifugation, and resuspended in DMEM for transplantation to recipient testes. Total cell count was determined before transplantation.

### ***Immunocytochemical localization of c-kit receptor and cytokeratin***

Isolated cells were centrifuged at  $30 \times g$  for 5 min onto glass slides in a Cytospin centrifuge (Shandon, Cheshire, UK). The cells were fixed and permeabilized with ice-cold acetone for 3 min and then washed 3 times with PBS containing 0.5 % Triton X-100 and 10% rabbit serum. Endogenous peroxidase activity was blocked with 0.1 - 1%  $H_2O_2$  for 5 - 10 min at room temperature. Blocking of nonspecific antibody binding was performed using 10% nonimmune rabbit serum. Cells were then incubated at 37 °C for 1 hr with 1:500 dilution of mouse monoclonal anti *c-kit* antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in PBS, the cells were incubated with 1:50 dilution of Horse Radish Peroxidase (HRP)-conjugated anti mouse IgG as secondary antibody (Sigma) for 45 min. After washing with rinsing buffer, 10% 3, 3'-Diaminobenzidine (DAB) solution (Sigma) was applied for 10 min. For the negative controls, the experiments were repeated but without using the primary antibodies. For cytokeratin immunocytochemistry, cells were fixed for 10 min in ethanol-acetone (1:1) at -20 °C rinsed with PBS and incubated for 10 min with 3%  $H_2O_2$  in PBS to quench endogenous peroxidase activity. Cells were washed for 30 min with PBS containing 0.2% bovine serum albumin (BSA), followed by 30 min incubation in 1% BSA in PBS and then incubated with the primary antibody for 1 hr. Mouse monoclonal anticytokeratin Pan (Boeringer-Mannheim, Germany) antibody was used as primary antibody. The secondary antibody was a peroxidase conjugated anti mouse IgG (Sigma) which was incubated for 90 min. After washing with rinsing buffer, 10% DAB solution was applied for 10 min. For the negative controls, the experiments were repeated but without using the primary antibody.

### ***Cytochemical detection of alkaline phosphatase***

Cells were removed from the seminiferous tubules by sequential enzymatic digestion and testis's tissue cryosections (positive control)

were evaluated for their expression of alkaline phosphatase after 3 days in culture.

Briefly, the cells were fixed *in situ* with ice-cold acetone for 2 min and washed twice with 0.2 M Tris buffer (pH= 8.74). The substrate reagent, prepared fresh, consisted of 0.01% naphthol-AS-MX phosphate and 0.06% Fast Violet salt (both from Sigma) in 0.1 M Tris buffer (pH= 8.74). This solution was immediately filtered and incubated with the fixed cells for 30 min at 37 °C. After incubation, the cells were washed with distilled water and observed under light microscope for a red bright color indicating the expression of alkaline phosphatase against a yellow background.

### ***Detection of 5-Bromo-2 deoxyuridine (BrdU) incorporation in spermatogonial stem cells***

The mixed population of the cells which were obtained by enzymatic digestion of testis's samples was placed on microscopic cover slip in 12-well tissue culture test plate and DMEM medium containing 10 % fetal bovine serum (FBS) was added. When the cells grew to about 70% confluence (proliferation phase), BrdU incorporation was performed by adding 0.1 mM BrdU to the culture medium for 24 hr. The BrdU incorporated cells were centrifuged at  $30 \times g$  for 5 min onto glass slides in a cytospin centrifuge and fixed in ice-cold acetone for 2 min. 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. After rinsing, the cells were placed in 0.1 M borate buffer (pH= 8.5) for 10 min for acid neutralization. Endogenous peroxidase activity was blocked with 1%  $H_2O_2$  and 10% goat serum for 15 min at 37 °C. Monoclonal anti BrdU antibody (Sigma) was diluted at 1:500 and applied to 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 as second antibody diluted at 1:50.

After extensive washing, 10% DAB solution was applied for 10 min. For the negative controls, the experiments were repeated but

without using the primary antibodies.

#### **Recipient mice and donor cell transplantation**

In the experiments with busulfan-induced degeneration of testicular spermatogonial stem cells, different amounts of busulfan (Sigma) were dissolved in Dimethyl Sulfoxide (DMSO; Sigma), and an equal volume of sterile distilled water was added to provide a final concentration of 15, 30, and 45 mg/kg of mice body weight. Adult mice (>6 wks of age) received a single intraperitoneal injection of busulfan. In each experiment, two to three animals received busulfan at each dose.

In the experiments with transplantation, busulfan (30 mg/kg) was used to deplete endogenous germ cells to prepare recipients. They were used as recipient mice for transplantation at least 6 wks after busulfan treatment. The donor cells were suspended in a volume of approximately 15  $\mu$ l of DMEM/FCS (fetal calf serum) and transplanted into left Rete testis of recipient with a microinjection needle. Viability of cells was greater than 95% as determined by trypan blue (Sigma) dye exclusion. For testicular injections, the cells (approximately  $10^6$ ) were maintained on ice and then microinjected into the seminiferous tubules using injection needle in one of the testes of each of the recipient mice; the other testis served as an internal control.

#### **Analysis of recipient testes**

The testes of busulfan-injected animals were recovered at 35 and 70 days after injection. The testes were fixed in 10% formalin (Merck, Darmstadt, Germany) and processed for paraffin sectioning. All sections were stained with hematoxylin and eosin. Two histological sections were made from the testes of each animal, and each slide was studied to determine the extent of spermatogenesis. The values for each time point were determined in at least three experiments and represented data from more than six testes. The number of tubule cross-sections showing spermatogenesis (defined as the presence of multiple layers of germ cells in entire circumference of the seminiferous tubule) or not showing spermatogenesis were recorded for

one section from each testis. For evaluation of spermatogonial stem cell colonization, recipient mouse testes were recovered 2 months after donor cell transplantation and analyzed by BrdU staining. This method allowed the specific identification of donor germ cells that had previously been incorporated with BrdU, because endogenous host testis cells did not stain with anti BrdU monoclonal antibody.

Transplanted testes were washed in PBS and then were placed in tissue frozen medium. This medium was frozen in liquid nitrogen for 2 min. Serial sectioning with 5  $\mu$ m thickness were prepared. The sections were then immunostained with a primary anti-BrdU in order to visualize the donor-derived spermatogenesis.

#### **Determination of testosterone levels and germ cells number**

Twenty four mice (C57BL/6) aged 15 days old were selected and divided into 4 equal groups (one control and three case groups). Animals derived from original stocks were purchased from Pasture Laboratory (Tehran, Iran). The groups 2, 3, and 4 received 2 subcutaneous injections of three different doses of hCG including 5, 10 and 50 IU respectively, on the days 15 and 25 of the mouse life. The level of serum testosterone was determined on days 28 and 65 of the mouse life. The mice were then killed and the different testicular cell populations were determined according to DNA contents by flow cytometric analysis.

Serum testosterone levels were measured using a radioimmunoassay kit (Immunotech, Marseille, France). For DNA flow cytometry, freshly harvested testicular tissues were separated from the tunica albuginea and minced in PBS. The resultant single-cell suspensions were washed twice in PBS, and 100  $\mu$ l aliquots of the suspensions were fixed with ethanol in PBS. After centrifugation, the pellets were resuspended in propidium iodide after subjection to RNA digestion by RNase (Sigma). DNA histograms were then obtained on a flow cytometer (Becton-Dickinson FAC scan, San Jose, Calif, USA). The data thus



obtained were analyzed by Cell Quest software (Becton-Dickinson).

After spermatogonial stem cell transplantation, a group of transplanted mice received 50 IU hCG and 2 months later were compared with uninjected group by evaluating *cyclin B1* expression.

#### Alkaline phosphatase assay

For examination of alkaline phosphatase activity the cells were fixed for 10 min in ethanol-acetone (1:1) and then incubated for 30 min in a solution containing 0.5 mg/ml Fast Blue RR (Sigma) and 40 µl/ml α-naphthol phosphate (0.25% solution, Sigma). After washing in water, the samples were mounted and examined.

#### Examination of capsular thickness and spermatogenesis regeneration

Fifty round testis tubules were randomly selected for each testis and the diameters of tubules and epithelium thickness were measured under light microscopy.

#### RNA extraction

RNA was extracted from the freshly isolated testis tissues by the AGPC (Acid Guanidium Thiocyanate-Phenol-Chloroform) method<sup>(17)</sup>. In brief, 400 µl RNA-Bee (Biosite, Stockholm, Sweden) solution was added per 0.5 mg fresh tissue, minced on ice using sterile scalpels and scissors and homogenized with a few strokes in a glass-Teflon homogenizer. Two hundred µl ice-cold chloroform (Merck) was added and the mixture was shaken strongly for 10 sec followed by centrifugation at 18000 × g for 10 min at 0 °C.

Upper aqueous phase was transferred very carefully, using a pipette to a new micro centrifuge tube. Equal volume of ice-cold isopro-

panol (Merck) was added to precipitate the RNA, followed by overnight incubation at -20 °C.

The tubes were centrifuged at 29000 × g for 15 min at 0 °C and the supernatants were discarded. The RNA precipitate was then washed by adding 500 µl of ice-cold 75% ethanol, and then centrifuged at 41000 × g for 10 min at 0 °C. The pellet was dried in air and resuspended in distilled water.

#### First strand cDNA synthesis and polymerase-chain-reaction (PCR) amplification

A semi-quantitative Reverse Transcription (RT)-PCR method was used to measure gene expression (*c-kit*, *cyclin B1*, and *oct-4*) in the testis tissue. Total RNA (1 µg) was heat-denatured at 65 °C for 5 min, and reverse-transcribed in the reaction mixture (20 µl) containing 5x Buffer (pH= 8.3; 50 mM Tris-HCl, 40 mM KCl, 6 mM MgCl<sub>2</sub>; Roche, Mannheim, Germany), 10 mM dNTP (Roche), N6 random hexamers (20 pmol/µl; Cyber Gene, Stockholm, Sweden), 200 U/µl M-MULV Reverse Transcriptase (Roche), and 2 µl distilled water at 42 °C for 1 hr. PCR amplification was done in a reaction mixture consisting of 10x PCR reaction buffer (pH= 8.3; 100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl<sub>2</sub>; Roche), 10 mM dNTP (Roche), the primers (10 pmol/µl), and Taq DNA Polymerase (5 U/µl; Roche). All primers were purchased from Cyber Gene and are listed in table 1.

Each cycle consisted of denaturation for 1 min at 94 °C, annealing (according to table 1) for 30 sec and extension for 30 sec at 72 °C. Extension during the last cycle was prolonged to 7 min. After PCR amplification, 10 µl of PCR products were applied to 2% ethidium

Table 1. Primer sequences and annealing temperatures used for RT-PCR

Genes	Direction	Sequences	Annealing temperature	Thermocycling
<i>mGAPDH</i>	Fwd	5'-CAGGAGCGAGACCCCACTA-3'	60 °C	30 cycle
	Rev	5'-GGCATGGACTGTGGTCATGA-3'		
<i>Oct-4</i>	Fwd	5'-GGCGTTCTCTTTGGAAAGGTGTTTC-3'	60 °C	35 cycle
	Rev	5'-CTCGAACCACATCCTTCTCT-3'		
<i>c-kit</i>	Fwd	5'-CATCCATCCATCCAGCACAA-3'	52.3 °C	35 cycle
	Rev	5'-CATCACACTCGATGAGGGAA-3'		
<i>cyclin B1</i>	Fwd	5'-AGTGCCTCTGAAAAGGGAAG-3'	53.3 °C	35 cycle
	Rev	5'-CTTCCTCCAGTTGTCCGAGA-3'		

Fwd, Forward; Rev, Reverse

bromide stained agarose gels for electrophoresis. To compare the expression levels of *c-kit* and *cyclin B1* genes, PCR products of the house keeping gene glyceraldehyde 3-phosphat dehydrogenase (*GAPDH*) and the specific genes (*c-kit* or *cyclin B1*) were loaded in the same well synchronously. Due to difference in the amplicon size, the PCR bands were separated and the strengths of the bands were analyzed by the Lab Works software (UVP, Upland, CA, USA).

#### **Analysis of *c-kit* protein expression**

Protein extracts of testis's cells collected from 6 weeks old (control samples) and busulfan-treated mice were used for western blot analysis. Total protein lysates were prepared by homogenizing the testes in ice cold RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF (Phenyl Methyl Sulfonyl Fluoride), 10 mg/ml of leupeptin, 10 mg/ml of aprotinin, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM NaF; Sigma). Lysates were cleared by centrifugation for 30 min at 20000 × g. Protein levels were measured using Bradford assay. Proteins were separated using SDS-PAGE and blotted onto a nitrocellulose membrane (Sigma). After transfer was completed, the membrane was stained with Ponceau S (Merck) to estimate transfer efficiency.

Western blots were blocked using PBS containing 2 % BSA. The membrane was incubated in 1 % BSA solution containing 1:500 dilution of primary antibody (*c-kit* monoclonal antibody, Santa Cruz) for 90 min at room temperature with gentle shaking. After 3×10 min washing in PBS Tween, incubation with 1:10,000 diluted HRP conjugated rabbit anti mouse immunoglobulins (Sigma) was performed for 30 min. For band detection, DAB solution was added and incubated for 10 min with gentle shaking.

#### **Statistical analysis**

Statistical analysis was done using SPSS, version 11.5, statistical software program. The Kruskal-Wallis test was applied for comparison of control and treatment groups

and Mann-Whitney Rank Sum test was applied for comparison of treatment groups with the control and p values less than 0.05 were considered statistically significant.

## **Results**

### **Regeneration of spermatogenesis after busulfan injection**

Morphological analysis of mouse testis structure following busulfan treatment showed that mortality was dose dependent and 90 mg/kg was the lethal dose for mice. In agreement with the results reported previously<sup>(18)</sup>, injection of busulfan decreased the testis size and weight and depleted spermatogenesis in a dose-dependent manner (Figures 1a and b). Morphological evaluation at 35 days after treatment revealed that, whereas only a few of seminiferous tubules showed spermatogenesis in animals treated with busulfan at doses of 45 and 90 mg/kg, spermatogenesis was observed in 15% and 53% of the tubules of animals given busulfan at doses of 30 and 15 mg/kg, respectively (Figures 1c-e). These observations indicated that the effects of busulfan were dose dependent.

Seventy days after busulfan treatment, the increase in weight of the testis was most apparent in animals treated at a dose of 15 mg/kg, suggesting extensive regeneration of spermatogenesis at this dose. Histological analysis showed that most testes contained no germ cells in animals treated with busulfan at 45 mg/kg, and that regeneration occurred in a few tubules.

On the other hand, regeneration of spermatogenesis was more significant in animals given the drug at 15 and 30 mg/kg, with averages of 80% and 35% of tubules showing spermatogenesis, respectively. Particularly in animals treated with busulfan at 15 mg/kg, virtually all seminiferous tubules showed apparently normal spermatogenesis with mature spermatozoa, and the size of the testis was almost comparable to that in uninjected controls. These results indicated that a significant proportion of spermatogonial stem cells were eliminated at 45 mg/kg, and that

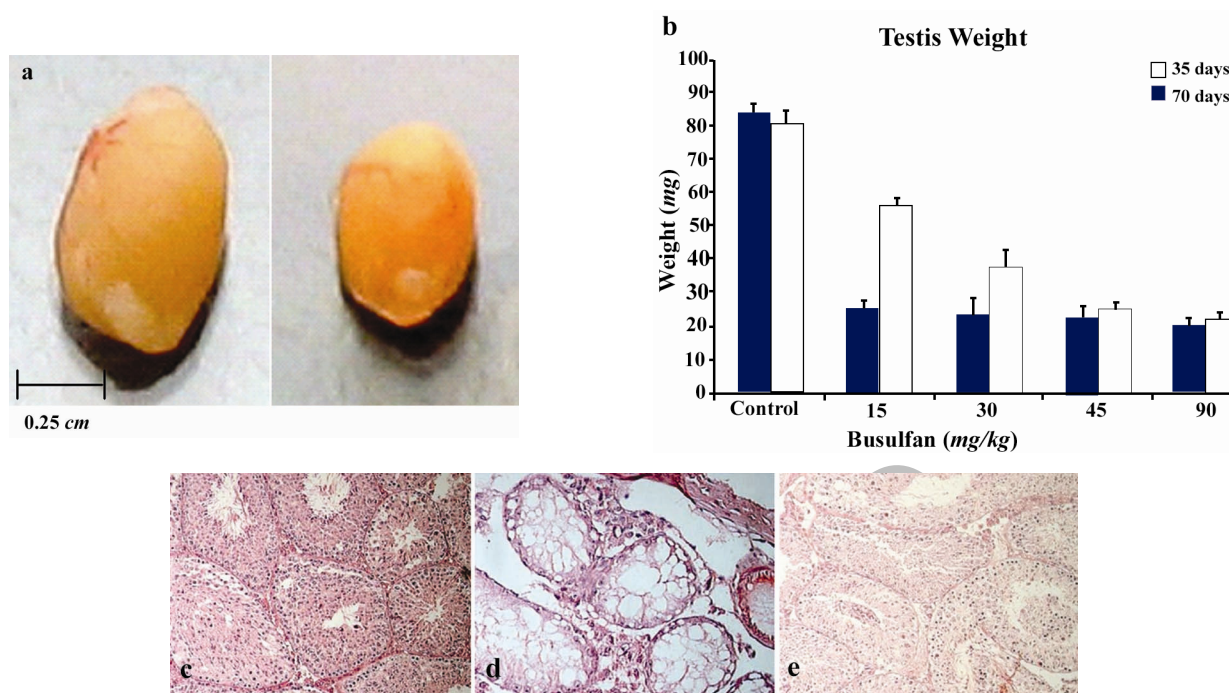


Figure 1. Regeneration of spermatogenesis after busulfan injection (a) Changes in testis size after injection of busulfan. Busulfan decreased the testis size and depleted spermatogenesis. (b) Changes in testis weight after injection of busulfan at various doses. The values for each time point were determined in at least three experiments and represent data from more than six testes. The increase was most significant after busulfan injection at a dose of 15 and 30 mg/kg. Values are means $\pm$ SD. Asterisks indicate significant statistical increase ( $p < 0.01$  by Kruskal-Wallis test). (c), (d), and (e) Histological changes of the testes that received busulfan injection at a dose of 30 mg/kg. (c) Wild-type B6 Testis (control). (d) Busulfan-treated B6 testis, 35 days after injection. (e) Busulfan-treated wild-type B6 testis, 70 days after injection. Note the increased number of seminiferous tubules with spermatogenesis after 70 days. Bar = 30 mm

surviving stem cells could regenerate to show spermatogenesis following injection of 15 and 30 mg/kg of busulfan. We selected a dose of 30 mg/kg for further analysis, because the stem cell regeneration, as suggested by the testis weight and the number of repopulated tubules, occurred extensively at this dose, but there are enough spaces for cell injection.

### Immunostaining

Spermatogonial stem cells express *c-kit* and we used it for confirmation of stem cells presence in colonies (Figure 2a). Therefore *c-kit* immunoreactivity as a molecular marker for spermatogonial cells was positive for the cells (Figure 2b). Figure 2c shows positive staining of Sertoli cells with anti-cytokeratin antibody throughout the cytoplasm. BrdU was added to the donor cells 2 days before transplantation and staining was examined just before transplantation. More than 80% of all the cells were labeled with BrdU before transplantation (Figure 2d).

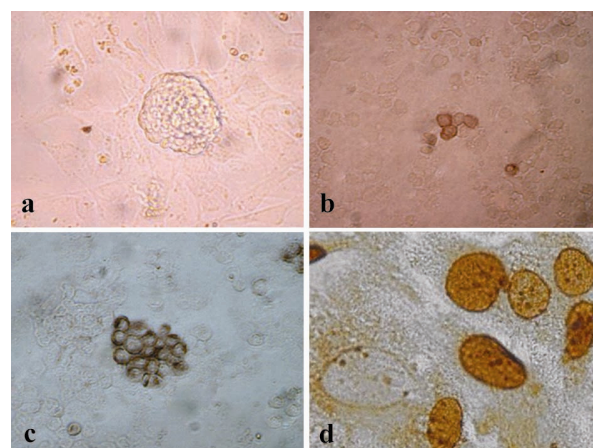


Figure 2. Spermatogonial stem cell detection (a) The morphology of spermatogonial-derived colony and spermatogonial stem cell proliferation and colonization. (b) C-kit immunoreactivity was positive for the spermatogonial stem cells. (c) Cytokeratin was detected in the neonatal Sertoli cells. (d) Spermatogonial stem cells were positive with monoclonal anti-BrdU antibody in culture medium. Magnification:  $\times 20$  (in a),  $\times 40$  (in b and c), and  $\times 100$  (in d)



Two months after transplantation, the cells showing nuclear BrdU staining were considered as transplanted cells. Transplanted spermatogonial cells resulted in full spermatogenesis in the recipient mouse testes. The right testis served as control group.

Alkaline phosphatase is highly expressed in mouse intestine (brush border of villous), as well as in adult and neonatal testes (Figures 3a-c) that served as positive control group in our study, but it is not expressed in spermatogonial stem cells (Figure 3d).

#### ***Capsular thickness examination and spermatogenesis regeneration of the control and busulfan treated mice testes***

Testis capsular thickness was evaluated by optical microscopy and was increased after busulfan treatment. Capsular thickness increase and tubular diameter reduction depended on busulfan dosage. There was no significant difference in capsular thickness and tubular diameter between doses 15, 30, and 45 mg/kg, but there was a significant difference in these parameters when the control group was compared with the experimental groups regardless of dosage of busulfan (Figures 4a-d).

#### ***Transplantation efficiency***

Two months after transplantation of cells onto seminiferous tubules of left testes, spermatogenic cells, as detected by tracing BrdU incorporated cells, were found in the

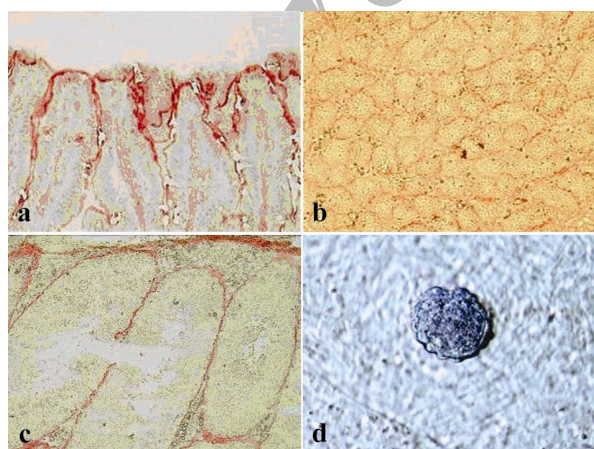


Figure 3. Alkaline phosphatase reactivity. Control groups including (a) mouse intestine, (b) adult and (c) neonatal mouse testis were alkaline phosphatase positive. (d) The colony did not show alkaline phosphatase reactivity. Magnification:  $\times 20$

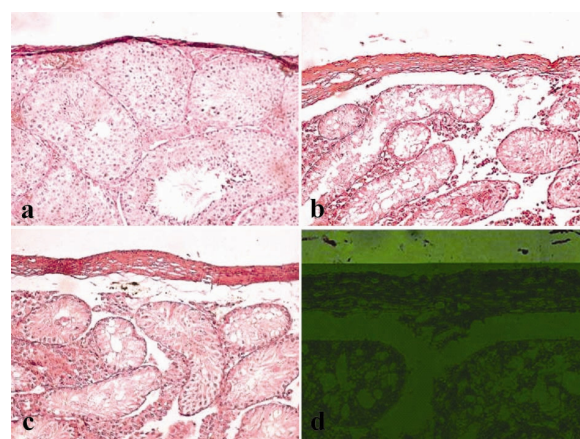


Figure 4. Histological examination of capsular thickness of mouse testes following busulfan treatment (a) Capsular thickness in normal testis (control), (b) 15 mg/kg busulfan treated testis, (c) 30 mg/kg busulfan treated testis, and (d) 45 mg/kg busulfan treated testis. The results showed that testis capsular thickness was increased after busulfan treatment in a dose-dependent manner. Magnification:  $\times 20$  (in a, b, and c) and  $\times 40$  (in d)

tubule cross-sections of recipient mice left testes (Figure 5a). No BrdU positive cells were found in the non-transplanted group (right testis which was considered as control group), confirming the specificity of this identification method (Figure 5b).

#### ***hCG effect on testosterone level and cell number***

Day 28 testosterone was shown to have increased as compared to the control group and the highest testosterone values were detected in group 4 (Table 2). In contrast to day 28, different results were found for day 65. In this regard, the testosterone levels were declined with increasing dose of hCG in different groups in comparison to the control group. Accordingly, group 4 had the lowest level of testosterone (Table 3). There was also no statistically significant difference detected

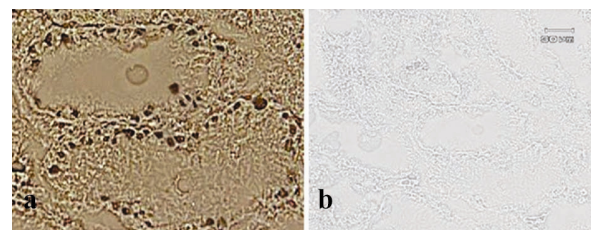


Figure 5. BrdU staining was performed with anti-BrdU antibody following spermatogonial stem cell transplantation (a) Spermatogonial stem cell detection in transplanted testis cryosection staining. (b) Negative control of BrdU Staining in testis tissue. Magnification:  $\times 60$



Table 2. Serum testosterone levels in different days and doses

Experimental group	Testosterone levels (nmol/l) SD ± mean	p-value
Control	0.2 ± 0.61	--
5 IU hCG	1.18 ± 1.64	NS
10 IU hCG	1.3 ± 2.62	NS
50 IU hCG	3.56 ± 4.75	p < 0.05

Serum testosterone levels on days 28. The highest testosterone values were detected in group 4, NS= Not Significant

between the case groups and the control group. Additionally, DNA flow cytometric analysis revealed that the germ cell numbers had remarkably declined in the case groups in comparison with the control group on day 65 and this reduction was statistically significant for groups 3 and 4 (Table 4).

Table 3. Serum testosterone levels in different days and doses

Experimental group	Testosterone levels (nmol/l) SD ± mean	p-value
Control	7.74 ± 2.78	--
5 IU hCG	0.81 ± 1.00	NS
10 IU hCG	3.1 ± 2.14	NS
50 IU hCG	0.57 ± 0.73	NS

Serum testosterone levels on days 65. The group 4 had the lowest level of testosterone level, NS= Not Significant

Table 4. Serum testosterone levels in different days and doses

Experimental group	Haploid cell percent SD ± mean	p-value
Control	2.38 ± 70.67	--
5 IU hCG	4.70 ± 68.46	NS
10 IU hCG	13.12 ± 55.67	p < 0.05
50 IU hCG	12.32 ± 36.69	P < 0.05

Percentage of haploid cell population of testis on days 65. DNA flow cytometric analysis revealed that the germ cell numbers had remarkably declined in the case groups in comparison with the control group on day 65. Data presented as the mean ± SD, NS= Not Significant

#### **Oct-4 gene expression in stem cell**

Our results revealed that *Oct-4* was exclusively expressed in testis cells of 3-6 days old mice and adult mouse testis and testicular cells from busulfan treated mice did not express *Oct-4* (Figure 6a). In adult mouse testis, spermatogonial stem cell differentiation resulted in down regulation of *Oct-4* expression and its transcript was not detected in RT-PCR gel electrophoresis.

#### **Evaluation of *c-kit* and *cyclin B1* gene expression by semi-quantitative RT-PCR**

We evaluated the expression of these genes in adult (control group), 3-6 days old, and busulfan treated (experimental groups) mouse testes by semi-quantitative RT-PCR. During proliferation of testis cells, expression rates of *c-kit* and *cyclin B1* was increased and thus these expressions in adult mice was more than that in neonatal testis. Busulfan eliminates most germ cells from the testis and destroys seminiferous epithelial leading to Sertoli cell disappearance. Germ cell reduction results in *c-kit* and *cyclin B1* expression decrease in adult busulfan treated mouse testis (Figures 6a and b). Using semi-quantitative RT-PCR analysis, expression rate of each gene was compared with *GAPDH* expression and *cyclin B1/ GAPDH* and *c-kit/ GAPDH* ratios were evaluated.

#### **Evaluation of *cyclin B1* gene expression after spermatogenesis induction in busulfan treated mice**

After hCG injection in stem cell transplanted mice, *cyclin B1* expression was evaluated by semi quantitative RT-PCR. Results revealed that *cyclin B1* expression increased after hCG injection. High quantity of gene expression showed that spermatogonial stem cell colonization and endogenous stem cells number increased during hCG injection (Figure 6c).

#### **SDS-PAGE/Immunoblot analysis of *c-kit* expression in spermatogonial stem cells**

The presence of *c-kit* protein in the testis was determined by SDS-PAGE/ immunoblot analysis. Total proteins (10 µg) from testis lysate of control and busulfan treated mice were separated by SDS-PAGE, transferred to Nitrocellulose membrane (NC) and probed with anti-*c-kit* antibody. The antibody recognized a single protein band of ~ 150 kDa in normal mice. No detectable *c-kit* protein was seen in busulfan treated testes, busulfan treatment caused complete depletion of germ cells, and thus these animals were used as recipients for germ cell transplantation (Figures 7a and b).

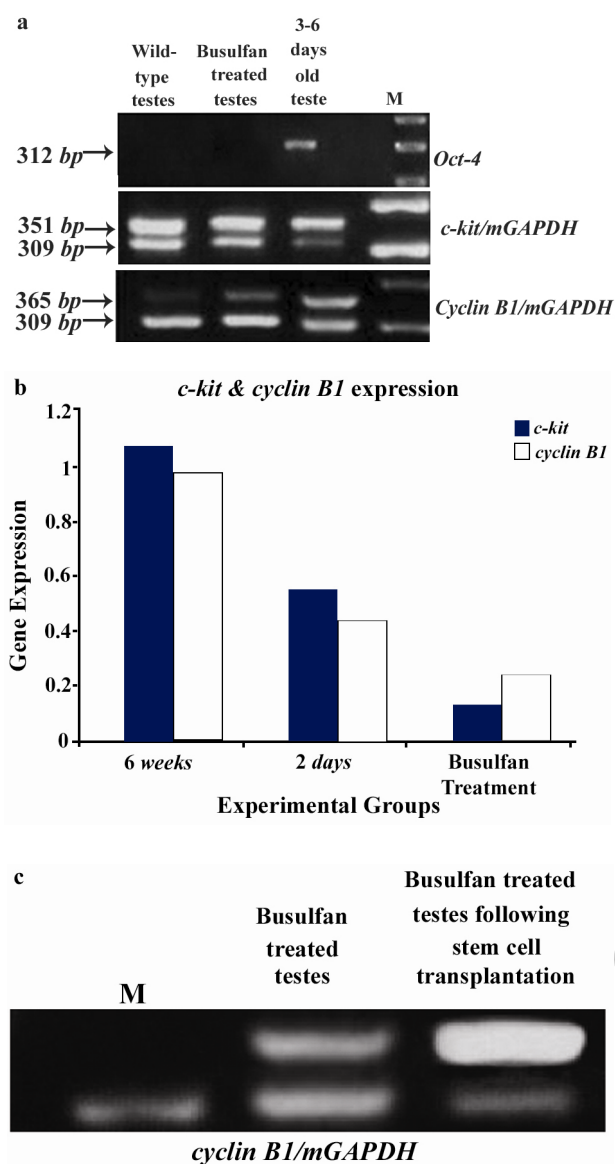


Figure 6. Evaluation of *Oct-4*, *c-kit*, and *cyclin B1* gene expression by semi-quantitative RT-PCR

(a) *Oct-4*, *c-kit*, and *cyclin B1* gene expression in spermatogonial stem cells. *Oct-4* was exclusively expressed in testis cells of 3-6 days old mice and adult mouse testis and busulfan treated mice did not express *Oct-4*. Germ cell reduction results in *c-kit* and *cyclin B1* expression decrease in adult busulfan treated mouse testis. (b) Results revealed that *cyclin B1* expression increased after hCG injection. M: Molecular marker (100 bp). (c) Busulfan eliminates most germ cells from the testis and results in *c-kit* and *cyclin B1* expression decrease in busulfan treated testes. Three-six days old testes have minimal proliferative action and these gene expressions

## Discussion

Biomedical researches have been boosted after stem cell discovery. The testis is among the organs that contain adult stem cells and proliferation and differentiation of these cells

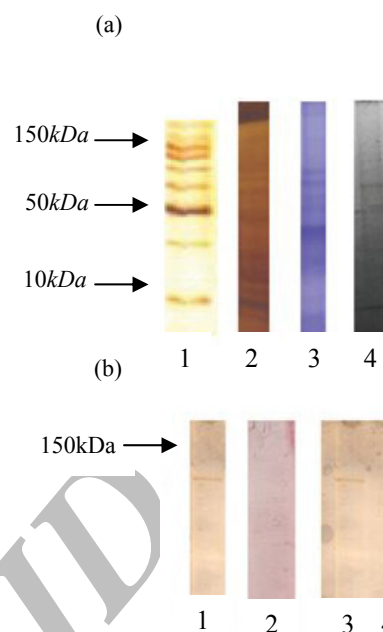


Figure 7. *c-kit* analysis in mouse testis

(a) SDS-PAGE. Lane 1: Molecular marker, Lane2: Normal testis (control) with AgNO<sub>3</sub> staining, Lane3: Normal testis (control) with Coomassie Blue staining, Lane4: Busulfan treated mouse testis. (b) Western blot. Lane1: Normal testis (control) Immunoblot, Lane2: Busulfan treated testis immunoblot, Lane 3 & 4: Normal & busulfan treated immunoblot synchronously. No detectable *c-kit* protein was seen in busulfan treated testes

play an important role in continuation of spermatogenesis<sup>(19)</sup>. Research on spermatogonial stem cells can provide the scientific community with valuable information about the process of spermatogenesis. Using these cells may enlighten the future of infertility treatment<sup>(20)</sup>. By time, some of the unaffected stem cells undergo spontaneous regeneration which may have important application in spermatogonial stem cell transplantation<sup>(21)</sup>.

This study revealed that increase in germ cell population leads to stem cell expansion that appears following busulfan treatment. Previous morphological studies have contributed to form the basic concept of stem cell self-renewal<sup>(22)</sup>. Our study revealed that regeneration of spermatogonial stem cells after busulfan treatment increased the efficiency of transplantation.

The results of busulfan injection confirmed that regeneration after cytotoxic treatment is based on stem cell expansion. Removal of

endogenous germ cells by busulfan treatment of recipient testes had a significant impact on donor stem cell colonization efficiency. As busulfan destroys primitive germ cells that account for less than 1% of the total number of testis cells<sup>(23, 24)</sup>, most of the differentiated progenitor cells remain and continue differentiation with normal kinetics. However, the latter gradually mature and disappear by 35 days, owing to the absence of self-renewal activity<sup>(23, 24)</sup>. Therefore, as the stem cell numbers continue to recover, the number of differentiated germ cells decreases, and the ratio of stem cells to differentiated germ cells changes markedly during regeneration. Eventually, all progenitor cells must be derived from the stem cells that initially survived busulfan injection. These processes are reflected in the changes in the weight and cell recovery observed during regeneration. The renewed and the re-induction of spermatogenesis in the left testis can be used as a measure of evaluating regeneration of spermatogonial division.

In this regard spontaneous recovery of spermatogonial stem cells in the right testis makes this evaluation difficult as there is no specific markers for discrimination of the donor stem cells for the endogenous recipient stem cells which is usually performed using transgenic animals.

Since, in our study, long term analysis of spermatogonial regeneration was not necessary and the objective was to survey the effect of hormone administration as well as stem cell transplantation on cell proliferation, we chose to administer the simple and reliable technique of BrdU incorporation in spermatogonial stem cells instead of using transgenic animals.

The present study used a mouse model and demonstrated that hCG injection following stem cell transplantation and evaluation of *cyclin B1* expression, has increased the efficiency of transplantation. Our results showed that hCG leads to increase in the proliferation of stem cells that may be due to increase testosterone levels shortly after

injection. These results are in agreement with previous studies in rat<sup>(15)</sup>.

However, hCG administration to neonate mice may have adverse effects on germ cell population, androgen secretion and probably germ cell apoptosis. Furthermore, the effects of hCG administration on the testis were time dependent, i.e. early administration of hCG in neonate mice increased testosterone levels and the number of germ cells. However, late administration of hCG showed absolutely opposite effects. The terms *early* and *late* refer to the evaluation of cell number and testosterone levels in a short and long time after hCG administration, respectively. The adverse effect of hCG on germ cells is probably due to the androgen withdrawal. Administration of hCG stimulates androgen production by the prepubertal testis<sup>(25)</sup>.

Following withdrawal of hCG injections, there is no further stimulation of the Leydig cells resulting in dramatic reduction in androgen production. Some data indicate that the drop in serum testosterone levels also causes apoptosis in testis germ cells, especially among cells<sup>(26)</sup>. Since serum testosterone levels are increased during hCG treatment, followed by a decrease, to levels as low as the prepubertal levels within a few weeks<sup>(27)</sup>, the increase in germ cell apoptosis after hCG treatment seen in this study most likely reflects the androgen withdrawal effect.

Few researches have studied the side effects of hCG treatment on testis. One investigation reported an increase in intravascular leukocyte aggregation and an increase in volume density of testicular blood vessels as well as the occurrence of interstitial bleeding, in the testes of hCG treated boys<sup>(4)</sup>. Along these lines, previous studies have indicated that hCG causes inflammation-like changes in both human and animal testes<sup>(28)</sup>. In this regard, treatment with hCG may be followed by an increase in germ cell apoptosis.

In the present study, only reduction in testosterone levels in group 4 mice after 65 days was related to a reduced cell population.



Therefore, mild androgen withdrawal may be safely tolerated by the germ cells. The mechanism by which hCG results in reduction of androgen levels is unknown. It may be caused by the fact that Leydig cells are also sensitive to androgen withdrawal; however, additional studies are necessary in this regard.

### Acknowledgement

We greatly appreciate the collaboration of Dr. A. S. Tabatabaei Panah, Dr. E. Javadi and Dr. H. Soltanghorae for their helpful discussions. We would also like to thank Dr. H. Zeraati for the statistical analyses and G. E. Kazemisefat, G. Ghasemi, M. Mobaraki, and M. Rashedan for their excellent technical assistance.

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