Original Article

Stem Cell INVESTIGATION

# Evaluating the behavior of cultured sertoli cells in the presence and absence of spermatogonial stem cell

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**Background:** The complex process of spermatogenesis is regulated by various factors. Several studies have been conducted to proliferate cells involved in the spermatogenesis process, in culture by used growth factors, different hormones and feeder cells. This study was conducted to evaluate the role of Sertoli cells on gene expression of fibroblast growth factor (*FGF2*) and glial cell derived neurotrophic factor (*GDNF*) after removal of spermatogonial stem cells (SSCs) from the culture medium.

**Methods:** Following isolation, bovine SSCs were co-cultured with Sertoli cells and follicular stimulating hormone (FSH) for 12 days. In the treatment group, SSCs were removed from the culture medium; in the control group no intervention was done in the culture. Colony formation of SSCs was evaluated by using an inverted microscope. Then, the expression of factors genes were assessed by quantitative RT-PCR. Data was analyzed by using paired-samples *t*-test.

**Results:** The results showed that removal of SSCs led to the increase in expression of *GDNF* and *FGF2*. These findings suggest that loss of SSCs population or decline in its population leads to changing in behavior of somatic cells which forming niche and consequently stimulates self-renewal and inhibits differentiation of SSCs.

**Conclusions:** The present study showed that removal of SSCs from the culture medium could be a model for damage to SSCs; the results revealed that niche cells respond to SSCs removal by upregulation of *FGF2* and *GDNF* to stimulate self-renewal of SSCs and abrogation of differentiation.

**Keywords:** Calves; gene expression; sertoli cells; spermatogonial stem cell (SSC)

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## Introduction

Spermatogonial stem cells (SSCs) are undifferentiated cells which are highly reproducible and expandable. These cells can generate completely similar cells (self-renewing); they can be differentiated and generate mature spermatozoa (1). It was recently shown that stem cells including SSCs are required to be in a specific tissue to express specific genes (2). In seminiferous tubules, SSCs are in near contact with Sertoli cells; sertoli cells produce suitable growth factors such as stem cell factors, transforming growth factor alpha and beta (*TGF-β*, *TGF-α*), insulin like growth factor1 (*IGF1*), fibroblast growth factor (*FGF*), epidermal growth factor (*EGF*) as well as hormones which regulate growth of male reproductive structures .In this context, *GDNF* and *FGF2* are of the most important growth factors that play pivotal role in regulation of SSCs development (2,3). *GDNF* is a member of *TGF-β* family that contributes to SSCs self-renewal in different species of mammals including bovine (1,2,4). *FGF2* stimulated germ cells proliferation and SSCs

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self-renewal *in vitro* (4) as well as *in vivo* (3). The removal of SSCs could help us know more about mechanisms by that niche cells to rebuild the germ cells after testicular damage, which could improve therapies to remake testicular germ cell in injury of testicular and germ cell evacuation (5). Accordingly, in the present research, the role of Sertoli cells on gene expression of *GDNF* and *FGF2* during removal of SSCs from the *in vitro* culture was assessed (6). Hormonal control of spermatogenesis is through FSH and testosterone activity on Sertoli cells. FSH is an essential prerequisite for maintaining spermatogenesis in adult mammals (7,8). FSH stimulates *GDNF* production by the Sertoli cells and consequently increases the SSCs self-renewal (9).

## Methods

The research was conducted in accordance with guidelines of the Animal Ethics Committee at the University of Tehran. The present study was conducted in the Department of Theriogenology, Faculty of Veterinary Medicine during February 2016.

### *Animals and testicular biopsy*

Testicular biopsies were obtained from Holstein bull calves (n=4), aged between 3 to 5 months, as previously used by our lab (10). Sedation with Xylazine and local anesthesia with lidocaine were performed. The testicular biopsies samples were transferred to the laboratory in 15 mL tube containing Dulbecco's Minimal Essential Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) with 10% Fetal Bovine Serum (FBS) (Sigma, USA) and 100 IU/mL penicillin-streptomycin (GIBCO, UK) on ice, within two hours.

### *Cell isolation*

Cell isolation was performed using a two-step enzymatic isolation procedure as previously used by our lab (11). Briefly, the obtained testis tissue was washed three times in DMEM containing antibiotics. They were then minced into small pieces as much as possible by a sterile scissor. Then they were suspended in DMEM containing 1 mg/mL collagenase (Sigma-Aldrich, USA), 1 mg/mL hyaluronidase (Sigma-Aldrich, USA), 1 mg/mL trypsin (Sigma-Aldrich, USA) and 5 µg/mL DNase (Fermentas, Germany) at 37 ℃ in a shaker incubator for 60 minutes. After three times washing in DMEM, the digested interstitial cells were removed and seminiferous tubules were remained. During the second step of enzymatic digestion, the seminiferous tubules were again incubated at 37 ℃ in DMEM containing 1 mg/mL collagenase, 1 mg/mL hyaluronidase and 5 µg/mL DNase for 45 minutes. In this step seminiferous tubules were deconstructed and their cells were separated. Finally, obtained cellular suspension was centrifuged at 30 ×g for 2 minutes to achieve population individual cells. Following filtration through 77 and 55 mm nylon filters, the cells were pelleted. The pellet was re-suspended in the DMEM containing antibiotics and 10% FBS.

### *Cell culture*

To evaluate gene expression, we used 6-well plates (TPP, Switzerland). Cells were seeded at concentration of $10\times10^5$ per well contacting DMEM and FSH (30 IU/mL). The plates were incubated at 37 ℃ in the presence of 5% $CO_2$ for 15 days. DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Sigma, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin was used for culturing cells. In the germ cell-removed group, the SSCs were removed from the *in vitro* culture as described by He *et al.* (12). Three hours after incubation, somatic cells attached to the bottom of wells but SSCs had remained in the suspension and consequently they were removed from the culture medium by using sampler (12) and no intervention was performed in the control group. In two groups, culture medium plus FSH were refreshed every 3 days.

### *Cells identification*

Vimentin is a cytoskeleton protein in Sertoli cell cytoplasm. At day 6 of culture, for Sertoli cells identification, Vimentin was stained, as described by Anway *et al.* (13) and Tajik *et al.* (14) and the specific marker Oct-4 was assessed in colonies of SSCs by the method proposed by Kubota *et al.* (4).

### *Evaluation of the colonization*

Colonies in each well were counted in the control and germ cell-removed groups were assessed using an inverted microscope (IX71, Olympus, Japan).

### *Gene expression*

Expression of the considered genes was assessed in the days 0, 6 and 12. Following trypsinization of the cultured cells (n=4 cell population from different calves), total RNA

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**Table 1** Sequence of the primers used for qRT-PCR

| Gene targets | Forward primer (3'-5') | Reverse primer (5'-3') |
|---|---|---|
| *B-ACTIN* | TCG CCC GAG TCC ACA CAG | ACC TCA ACC CGC TCC CAA G |
| *FGF2* | AAA ACA GGA CCT GGG CAG AA | ATA TAC CTC TTC ATG TAA AAT GAG ATC AGA TG |
| *GDNF* | GCAGCC GAA ACA ATG TAC GA | AAG GCG ATG GGT CTG CAA |
| *THY-1* | TTC ATC TCC TTG TGA CGG GTT | GCA GAG GTG AGG GAA TGG C |
| *c-KIT* | TAC CAA CCA AGG CAG ACA A | CTT TGA GGC AAG GAA CGC |
| *BCL6B* | AGG GCA CAG GGA ACT CTT TTC | CCT C CT TTG GCT TGA GTG TTTT |

qRT-PCR, quantitative real time polymerase chain reaction.

existing in the cells was extracted using Trizol (Fermentas, Germany). In order to prevent contamination of DNA, the extracted RNA was treated by DNase I (Fermentas, Germany). Concentration of the extracted RNA was determined by using spectrophotometry (Eppendorf, Germany). cDNAs were built by using 500 ng RNA extracted and oligo-primers and cDNA synthesis kit (Fermentas, Germany). *Table 1* lists the primers of the considered genes. PCR was done by using SYBR Green master mix (Fermentas, Germany) and by thermocycler (Applied Biosystems, USA). PCR started with a primary melting stage for 5 minutes at 95 ℃ to activate polymerase and continued with 40 cycles including melting (30 s at 95 ℃), synthesis (30 s at 58 ℃) and formation (30 s at 72 ℃). Quality of PCR reactions was determined by melting curve analysis. For each sample, PCR was done for reference gene (*B-ACTIN*) and target gene simultaneously. Cycle threshold (Ct) of the reference gene was subtracted from cycle threshold of the target gene to obtain ΔCt. In each interaction Ct on day zero was considered as a calibrator. Consequently, the relative gene expression was obtained by using Livak and Schmittgen (15) and calculation of ΔΔCt.

### *Statistical assessment*

Data were analyzed statistically by using SPSS, version 24. Gene expression data was analyzed by using paired-samples *t*-test. Data was reported in the form of mean ± standard deviation. Differences were considered significant ($P<0.05$).

## Results

### *Immunocytochemical staining of Sertoli cells and SSCs*

Presence of the vimentin in sertoli cells was shown by immunocytochemistry staining (*Figure 1*) and Oct-4 was detected in the colonies of SSCs (*Figure 2*).

### *Colonization of SCCs*

SSCs Colonies were developed in the control group (A) and no colony was observed in the germ cell-removed group (B) (*Figure 3*).

### *Gene expression*

Expression of FGF2 significantly increased in both groups on days 6 and 12 compared to day 0 ($P<0.0001$). On days 6 and 12, expression of *FGF2* was not different in group 1 and 2 ($P>0.05$). On days 6 and 12, expression of *FGF2* was significantly higher in group 2 than group 1 ($P<0.05$) (*Figure 4*).

In two groups expression of *GDNF* significantly increased on days 6 and 12 compared to day 0 ($P<0.05$), while expression of *GDNF* was not different on days 6 and 12 ($P>0.05$). On day 6, expression of *GDNF* was higher in group 2 than group 1 ($P<0.05$), while it was not different in group 1 and 2 on day 12 (*Figure 5*).

Expression of *THY1* significantly increased in group 1 on day 6 ($P=0.027$) and 12 ($P<0.0001$) compared to day 0 ($P<0.05$). Moreover, a significant increase was observed on day 12 compared to day 6 ($P=0.005$). Expression of *THY1* was not observed in group 2 (*Figure 6*).

In group 1, expression of *C-Kit* significantly decreased on days 6 and 12 compared to day 0 ($P<0.05$); however, no significant difference was found between days 6 and 12 ($P=0.396$). Expression of *C-Kit* was not observed in group 2 (*Figure 7*).

In group 1, expression of *BCL6B* was significantly higher on day 6 ($P=0.004$) and day 12 ($P<0.0001$) than day 0

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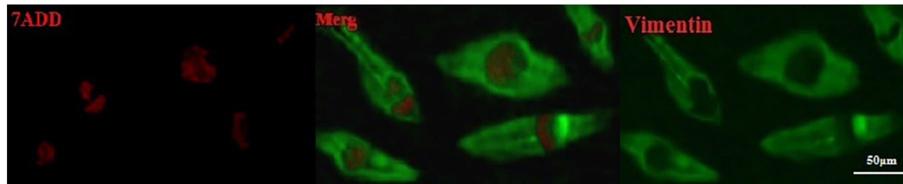


**Figure 1** Immunocytochemical staining of bovine sertoli cell for vimentin at day 6 of culture. 7ADD staining is used to demonstrate the nuclei of sertoli cells.

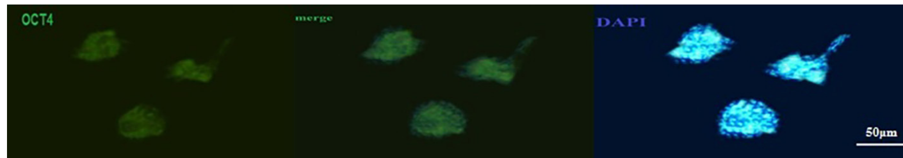


**Figure 2** Immunocytochemical staining of bovine SSCs for Oct-4 at day 6 of culture. DAPI is the nuclear staining of SSCs. SSCs, spermatogonial stem cells.

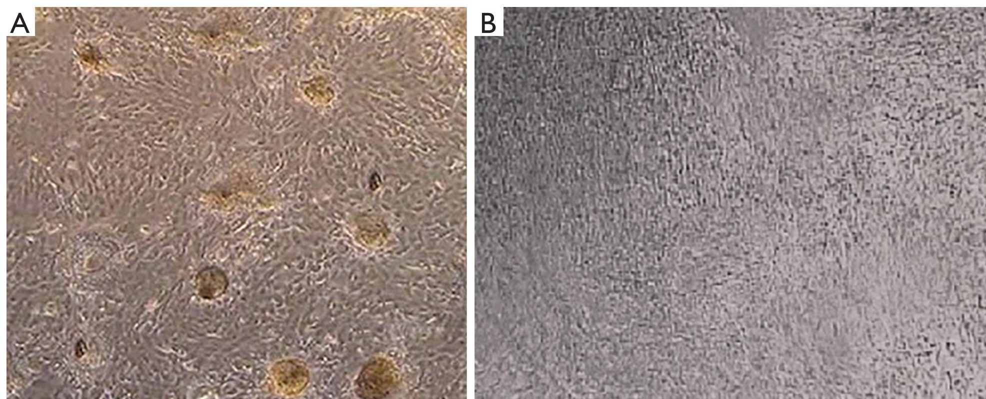


**Figure 3** Colonization SSCs of on day 12 in control (A) and germ cell-removed (B) groups (magnification ×400). SSCs, spermatogonial stem cells.

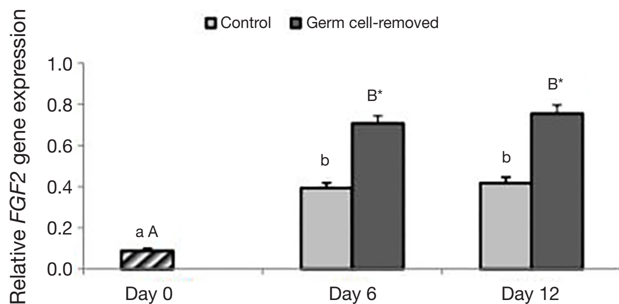


**Figure 4** Relative gene expression of *FGF2* in the control and germ cell-removed on days 0, 6 and 12. Small letters: indicate difference in Control group. Capital letters: indicate difference in germ cell-removed group. Different letters [(a, b), (A, B)] indicate significant difference in groups between in different time-points ($P<0.05$). *, indicates significant difference between two experimental groups at the determinate time-points ($P<0.05$).

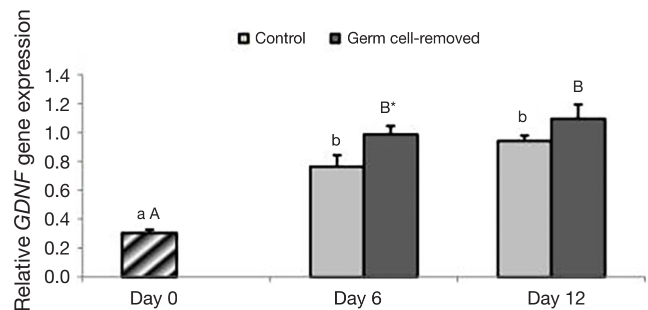


**Figure 5** Relative gene expression of *GDNF* in the control and germ cell-removed on days 0, 6 and 12. Small letter: indicate difference in Control group. Capital letter: indicate difference in germ cell-removed group. Different letters [(a, b) (A, B)] indicate significant difference in groups between in different time-points ($P<0.05$). *, indicates significant difference between two experimental groups at the determinate time-points ($P<0.05$). *GDNF*, glial cell derived neurotrophic factor.

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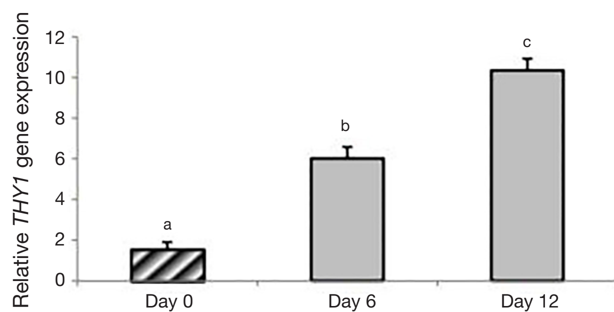


**Figure 6** Relative gene expression of *THY1* in the control group on days 0, 6 and 12. Different letters (a, b and c) indicate significant difference in different time-points (P<0.05). Expression of *THY1* was not observed in germ cell-removed group.

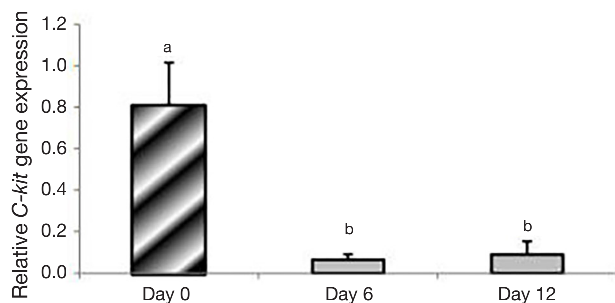


**Figure 7** Relative gene expression of *C-Kit* in the control group on days 0, 6 and 12. Different letters (a and b) indicate significant difference in different time-points (P<0.05). Expression of *C-Kit* was not observed in germ cell-removed group.

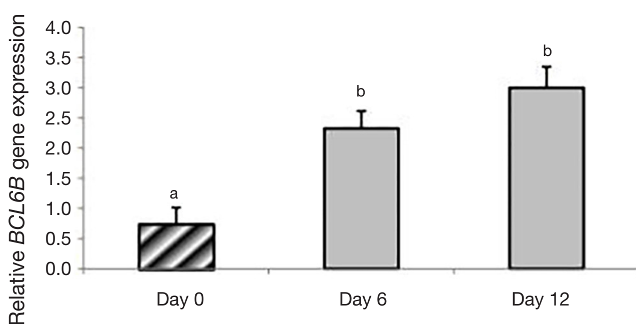


**Figure 8** Relative gene expression of *BCL6B* in the control group on days 0, 6 and 12. Different letters (a and b) indicate significant difference in different time-points (P<0.05). Expression of *BCL6B* was not observed in germ cell-removed group.

(P<0.05). There was no significant difference on days 6 and 12 in expression of *BCL6B* in group 1 (P>0.05). Expression of *BCL6B* was not observed in group 2 (*Figure 8*).

## Discussion

Colony formation, resulting from the interactions between SSCs and Sertoli cells during *in vitro* culture (16,17), were absent in the germ cell-removed group (18). In this context, *THY1* (2,4,18) and *BCL6B* (4,8) are considered as undifferentiated markers of spermatogonia and expression of these markers has been reported in rich populations of SSCs. On the other hand, *C-Kit* is known as differentiated spermatogonial marker (19). Expression markers of spermatogonia, covering *THY1*, *BCL6B* and *C-Kit* (20,21), was not observed in the germ cell removed group. These findings showed that removal of SSCs was successfully performed in the present study. Vimentin is a cytoskeleton protein in Sertoli cell cytoplasm. For Sertoli cells identification, Vimentin was stained. This finding is similar to the previous studies by Anway *et al.* (13). For confirmation of the presence of SSCs, the specific marker Oct-4 was assessed in colonies of SSCs by the method proposed by Kubota *et al.* (4). Hence, the present study showed that SSCs removal increased the expression of *GDNF* during the culture. The recent studies showed that, sharp increase in *GDNF* expression stimulates self-renewal and inhibits differentiation of SSCs (2,22). On the other hand, undifferentiated SSCs gradually disappear in mice with deficient *GDNF* gene expression and only Sertoli cells remain in seminiferous tubules (2). In addition, assessment of *GDNF* expression has shown that *GDNF* plays a basic role in proliferation and differentiation of SSCs (19).

Studies have shown that addition of *GDNF* to culture leads to proliferation of SSCs (4,12). Hence, self-renewal of SSCs in ordinary culture can be increase expression of *GDNF*, as previously reported by He *et al.* (12). Expression of GDNF increased in response to removal of SSCs from the culture medium; this phenomenon may indicate reaction of Sertoli cells to rebuild reserves of testicular stem cells, has been reported by Tadokoro *et al.* (9) and Zohni *et al.* (23). Busulfan treatment has direct consequences of SSC loss and expansion, the testicular somatic environment responds

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rapidly and temporarily to the loss of spermatogonia, by Increasing expression of *GDNF*, after treatment with busulfan (23). Busulfan preferentially kills spermatogonia but it doesn't affect on sertoli cell numbers. After busulfan treatment Sertoli cells have expressed *GDNF* in accordance the degree of damage on the spermatogonial population (24). Proliferation and self-renewal of SSCs stimulated by *FGF2* (3,4). *FGF2* and *GDNF* through upregulation of *ETS* variant have Synergistic effect (6) (*Etv5*), in addition *GDNF* signals increased expression of receptor tyrosine kinas Ret (3). Expression of *FGF2* increased in two groups during culture. Therefore, it seems that the increase in expression of *FGF2* is another reaction of Sertoli cells to rebuild testicular stem cells following the loss of testicular germ cells. This phenomenon, dominance of self-renewal on differentiation during the regeneration of germ cells, was previously observed in testis of mice treated with busulfan and lost a considerable part of their germ cells (25).

These finding could help develop therapies for acceleration of male fertility amendment after chemotherapy. Moreover it could improve *in vitro* condition for increase of self-renewal to SSCs transplantation. This research could be suggested that removal of SSCs could serve as a model to study the events ensuing testicular germ cell damage and the mechanisms involved in regeneration of testicular germ cells afterwards.

## Conclusions

The present study showed that removal of SSCs from the culture medium could be a model for damage to SSCs; this is followed by upregulation of *FGF2* and *GDNF* to stimulate self-renewal of SSCs and abrogation of differentiation. This reaction of somatic niche cells occurs to retrieve reserves of testicular germ cells in response to the damage.

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## Footnote

*Conflicts of Interest*: The authors have no conflicts of interest to declare.

*Ethical Statement*: The research was conducted in accordance with guidelines of the Animal Ethics Committee at the University of Tehran (7508016/6/25).

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