

## Comparison of colony formation of human spermatogonial stem cells (SSCs) with and without collagen

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### Abstract

**Objective:** To investigate the effects of collagen and growth factors on in vitro proliferation of human spermatogonial stem cells obtained from patients with non-obstructive azoospermia.

**Methods:** The experimental cross-sectional study was conducted from February 2013 to April 2015 after obtaining approval from the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Iran. Testicular sperm extractions of non-obstructive azoospermic patients were obtained from the Clinical Urology and Embryology, In Vitro Fertilization Department of Imam Khomeini Hospital.

Spermatogonial stem cells and Sertoli cells, obtained from human testis biopsies by a two-step enzymatic digestion method, were purified using fluorescence-activated cell-sorting and daturastramonium-lectin, and were cultured separately. To investigate a more direct influential factor on colony formation, one control and two experimental groups were formed. Group 1 acted as the control in which spermatogonial stem cells were co-cultured with Sertoli cells alone. In group 2 they were co-cultured with Sertoli cells and growth factors such as leukaemia inhibitory factor, epidermal growth factor and glial cell-derived neurotrophic factor, and in group 3 with Sertoli cells along with growth factors in the presence of collagen-coated dishes. Number and diameter of the colonies were evaluated after 7 weeks.

**Results:** Specimens obtained related to 21 patients. Number and diameter of the colonies in group 3 ( $18 \pm 2.6$  and  $276.6 \pm 45.5$ ) were significantly more than both groups 1 ( $3.5 \pm 1$  and  $D1:81.6 \pm 12$ ) and group 2 ( $11 \pm 2.2$  and  $165.2 \pm 32.5$ ) ( $p < 0.05$  each). Also, the number and diameter of colony in group 2 were significantly better than the control group ( $p < 0.05$ ). Expression profile of the VASA, promyelocytic leukaemia zinc-finger (PLZF), Octamer-binding transcription factor 4 (OCT4) and integrin  $\alpha 6$  (INTG $\alpha 6$ ) were detected in all groups. Based on cytochemical findings, OCT4 was expressed in the colonies of all three groups.

**Conclusion:** According to positive effects of collagen and growth factors on the colonisation of spermatogonial stem cells, it seems that using the cells may lead to better colonisation of this type of stem cells.

**Keywords:** Spermatogonial stem cells, SSCs, Growth factor, Collagen, Co-culture. (JPMA 66: 285; 2016)

### Introduction

Spermatogenesis is a highly complex and regulated process in which germ stem cells differentiate into spermatozoa. Spermatogonial stem cells (SSCs) play a key role in spermatogenesis and in vitro self-renewal; thus, safeguarding of SSCs is like pledging to restore fertility in patients with non-obstructive azoospermia.<sup>1</sup>

It was reported that some cancer treatment process, such as chemotherapy and radiotherapy can lead the patients to infertility due to loss of germ cells. Therefore, due to increase in the survival rate of cancerous patients after treatment, especially children, the treatment of infertility in these patients seems to be more important.<sup>2</sup> In pre-

pubertal boys who are not yet able to produce mature spermatozoa, in vitro maturation of SSCs is also an alternative option for spermatogonial maturation.<sup>3</sup>

However, generally the rate of SSCs in human testis is low<sup>4</sup> and the culture of these cells is difficult. In addition, a significant reduction in survival rate of the cultured cells happens during the first week.<sup>5</sup> To address these problems, several studies have been done.<sup>6-11</sup> Spermatogenesis is regulated by endocrine and autocrine growth factors, and a paracrine testicular factor that is produced by Sertoli cells, germ cells, peritubular and interstitial cells (Mainly Leydig cells and macrophages).<sup>12</sup>

In vitro propagation of human SSCs has been reported with small pieces of normal human testis,<sup>13</sup> as well as small testicular biopsies from azoospermic and non-obstructive azoospermic (NOA) patients with a number of important growth factors, including stem cell factor (SCF), leukaemia inhibitory factor (LIF), glial cell-derived neurotrophic factor (GDNF), basic fibroblast growth factor

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(bFGF) and epidermal growth factor (EGF) in different culture systems.<sup>8,13</sup>

Sadri et al. declared that using of laminin-coated dishes with combination of growth factors improves establishment and colony formation of SSCs.<sup>13</sup>

Collagens are a family of ubiquitous structural proteins found in extracellular matrix (ECM) of mammalian tissues, such as the testis. Type IV collagen, a network-forming n type,<sup>14</sup> and laminins are the most abundant building blocks of the basement membrane.<sup>14-17</sup> Collagen can take part in signal transduction via transmembrane receptors, such as integrins.<sup>16,18</sup> Moreover, it was shown that the non-collagenous (NC1) domain of collagen takes part in the regulation of adhesion, proliferation and apoptosis in various cell types via their interactions with integrins, such as  $\alpha\beta 1$ . These findings have clearly illustrated the signalling function of collagen.<sup>19</sup> To our knowledge, there is no report about the effects of collagen and growth factors on the proliferation of adult human SSCs.

The current study was planned to compare the in vitro effects of collagen and growth factors on the proliferation of adult human SSCs obtained from azoospermic patients.

## Materials and Methods

The experimental cross-sectional study was conducted from February 2013 to April 2015 after obtaining approval from the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Iran. Testicular sperm extractions (TESE) of NOA patients were obtained from the Clinical Urology and Embryology In Vitro Fertilization Department of Imam Khomeini Hospital with the informed consent of the subjects.

The germ cells were separated using a second enzymatic digestion. Briefly, TESE samples were transported into Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 13.5g/L, sodium bicarbonate (NaHCO<sub>3</sub>) (Sigma), single-strength non-essential amino acids (Invitrogen), 100IU/ml penicillin (Invitrogen), 100 $\mu$ g/ml streptomycin (Invitrogen), 40 $\mu$ g/ml gentamycin, and 5% foetal bovine serum (FBS) (Invitrogen). The tissue samples were washed three times with phosphate-buffered saline (PBS) solution supplemented with 1% penicillin-streptomycin-sulfate, and then placed in DMEM medium. Next, tissues were mechanically dissected by two insulin needles and dissociated in PBS. After two washes, the tissues were placed in enzyme solution containing 1mg/ml collagenase type I (GIBCO, Invitrogen), 1mg/ml hyaluronidase (Sigma), 1mg/ml trypsin (Sigma, St Louis, MO) and 0.05mg/ml deoxyribonuclease (DNase) (Sigma,

St Louis, MO) for 45 min at a temperature of 37°C. During this time some shaking and pipetting every 10 minute was done for efficient dissociation. After 45min, dissociated fragmented tubules, tissues and cells were centrifuged for 2 min at 1200 rounds per minute (rpm) and washed three times in DMEM medium. For the second digestion step, fresh enzymes were added to fragmented tubules and incubated for 30-45 minutes at 37°C. After filtration through 40 $\mu$ m nylon filter (BD Falcon), the cell suspension was centrifuged at 1500 rpm for 10 minutes.<sup>11,13</sup>

After obtaining the cell suspension from the TESE samples, the cells were re-suspended in DMEM/F-12 (Invitrogen) supplemented with 10% FBS, and Sertoli cells were extracted. Briefly, Petri dishes with a diameter of 60mm were coated with a solution of 5 $\mu$ gmL of daturastramonium agglutinin (DSA, Sigma) in PBS at 37°C for 1 hour.

Next, coated plastic dishes were washed three times with DMEM containing 0.5% bovine serum albumin (BSA) (Sigma). The cell suspension was placed on lectin-coated dishes and incubated for 1h at 37°C in a humidified atmosphere of 5% carbon dioxide (CO<sub>2</sub>) in air. Then, the floating cells were collected and washed twice with medium. After formation of a Sertoli cells confluent layer, Sertoli cells were detached by ethylenediaminetetra acetic acid (EDTA) - trypsin treatment for 5 min at 37°C. Next, the collected cells were counted and placed into Petri dish for secondary culture in DMEM at 37°C in the presence of 10% FBS (Gibco).<sup>20</sup>

After isolation of Sertoli cells, residual cell suspension was used for spermatogonial cell isolation. The media were collected, and the cells were gently washed twice with DMEM (Invitrogen). The Platte of cells was then washed once with PBS and the collected cells were centrifuged at 1500 rpm for 10 minutes, suspended in basic media. The single-cell suspension obtained was then incubated with 5% BSA (Sigma) in PBS for 30 minutes at 4°C, washed with 0.1% BSA in PBS, and incubated with an Alexa Fluor 488 1:100 conjugated antibody against the CD49f surface marker (Biolegend) on ice 1h. Peripheral blood lymphocytes were used as positive controls for CD49f, while human skin fibroblast feeder cells were employed as the negative control. Cells that were positive for CD49f were separated from the whole population by fluorescence-activated cell-sorting (FACS).<sup>21</sup>

Human CD49+SSCs were seeded at a density of 6000 cells in 4-well culture plates. Before culturing, cell count and viability were performed. All cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator. To investigate a

more direct influential factor on colony formation, one control and two experimental groups were formed with 23 samples in each group. Group 1 acted as the control in which SSCs were co-cultured with Sertoli cells alone. In group 2 they were co-cultured with Sertoli cells and growth factors such as LIF, EGF and GDNF, and in group 3 with Sertoli cells along with growth factors in the presence of collagen-coated dishes.

In groups 2 and 3 SSCs were cultured in knockout DMEM medium supplemented with recombinant human GDNF 10ng/mL (Peprotech), recombinant human LIF 10ng/mL (Peprotech) and recombinant human EGF 20ng/mL (Sigma). In all three groups knockout DMEM supplemented with 1mmol L-1L-glutamine, 0.1 mmol L-1  $\beta$ -mercaptoethanol, 1 mmol L-1 sodium pyruvate, 100  $\mu$ g mL-1 transferrin (Sigma).

Usually, three days after initial culture, medium was changed. To assess the proliferation rate, cells were passaged with trypsin-EDTA (0.25%), and were re-cultured or sub-cultured.

The number of colonies was determined using an inverted microscope and the diameter (d) of each colony was evaluated (Motic Software).

Immunocytochemical staining of the colonies was performed to identify octamer-binding transcription factor 4 (OCT4) as marker for SSCs. Colonies were washed three times with PBS and fixed in 4% paraformaldehyde (Sigma) for 20 minutes. The permeabilised cells were then incubated for 30 minutes at room temperature with 0.2% Triton X-100 (Sigma) and for blocked extraneous antibody incubating with 10% normal donkey serum for 1h was performed. After incubation, cells were washed three times with PBS and incubated overnight at 4°C with anti-OCT4 primary antibody (Santa Cruz) (1:100). Then cells

were washed three times with PBS and were incubated with the corresponding secondary antibody (1:100) for 1-2 h in the dark room. Next, the cells were washed with PBS and nucleus staining was done with 1  $\mu$ g/mL 4',6'-diamidino 2-phenylindole (DAPI) (Sigma) for 10 minutes. Finally, the cells were washed with PBS and observed with a fluorescent microscope (Olympus IX71). In every group one sample stained as negative control in that primary antibody did not add.

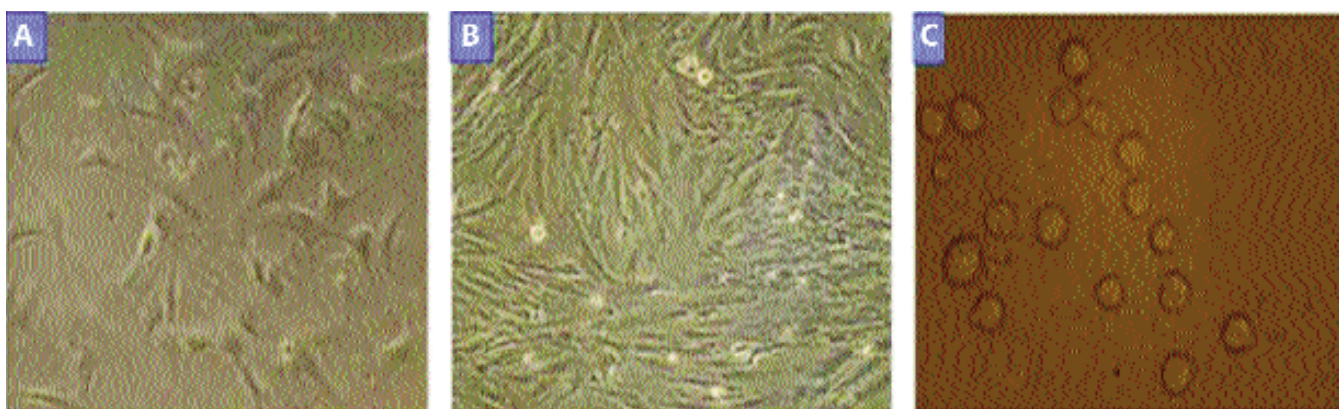
Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess the expression of spermatogonial cells genes, specifically OCT4, VASA, promyelocytic leukaemia zinc-finger (PLZF), and integrin  $\alpha$ 6 (INTG $\alpha$ 6), during the entire culture. Gene's expression was investigated in messenger ribonucleic acid (mRNA) level. For this purpose, the cells derived from colony were studied after 7 weeks. To perform RT-PCR, RNA extraction took place initially according to the Qiagen Kit and then their concentration was studied using an ultraviolet (UV) spectrophotometer.

In the next step, after the total RNA preparation and complementary deoxyribonucleic acid (cDNA) synthesis was performed using the Qiagen Kit. Then PCR of desired genes was performed using FERMENTAS Kit. Finally, agarose gel electrophoresis was done to check product quality RNA and PCR reaction product. Moreover,  $\beta$ -actin, housekeeping-gene, was included as an internal control (Table-1).

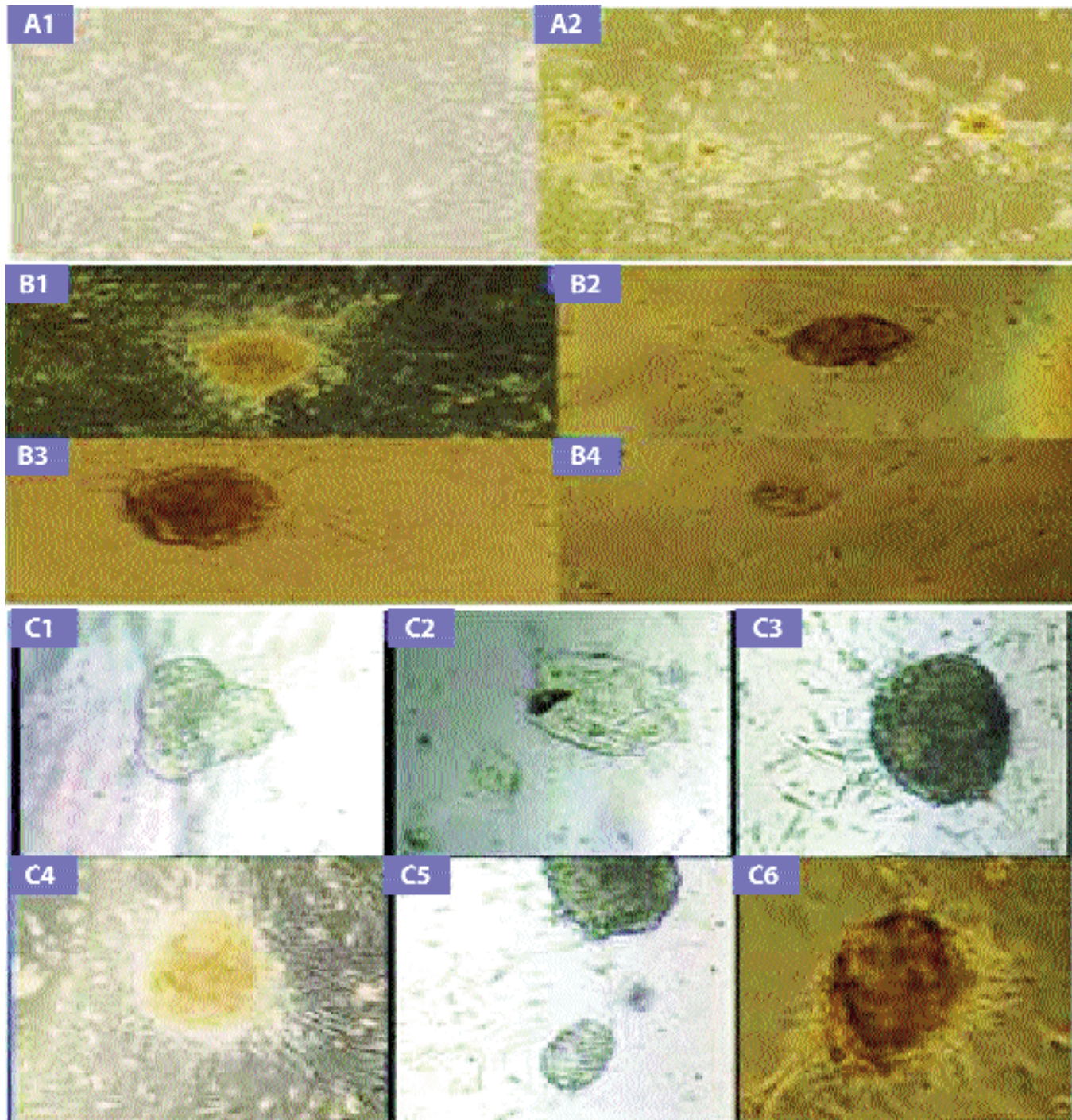
Results were expressed as mean  $\pm$  standard deviation. Data was analyzed using analysis of variance (ANOVA).  $P < 0.05$  was considered significant.

## Results

Specimens obtained related to 21 patients.



**Figure-1:** Sertoli cells: the cells population obtained from daturastramonium-lectin (DSA-lectin) isolation after 5 days (A) and at 7-10 days (B) and spermatogonial stem cells (SSCs) after isolation by fluorescence-activated cell-sorting (FACS) (C). Magnification:  $\times 60$ .

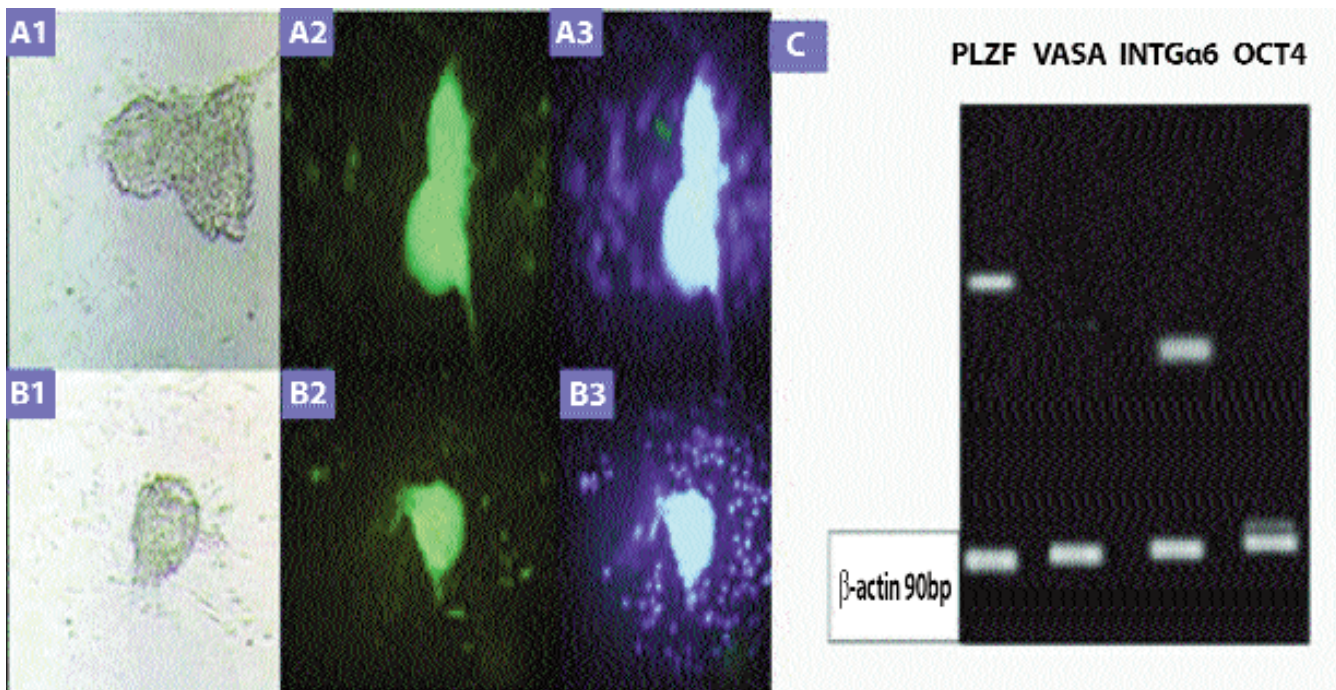


**Figure-2:** Morphology of spermatogonial stem cells (SCCs) and colonies during culture in control (A), growth factor (B) and growth factor plus collagen(C) groups. Magnification:×10(A1,A2,B4,C2,C5) , ×20(B1,B2,B3,C1,C3),×40(C4,C6).

After two-step enzymatic digestion, cell viability was assessed and approximately 88% cells were viable.

Sertoli cells Appeared between 24-36h after primary

culture. After 7-10 days, a monolayer of Sertoli cells appeared and this time CD49-positive cell suspension co-cultured with the Sertoli cell to provide environment that was similar in vivo as closely as possible(Figure1).



**Figure-3:** Characterisation of human spermatogonial stem cells(SSCs) that were cultured in knockout Dulbecco's modified Eagle's medium(DMEM) medium with growth factor plus collagen (A1) and growth factor group (B1) for 7-week.Magnification:  $\times 20$ . Plane A2 and B2 showed the expression of OCT4 in the colonies. 6'-diamidino 2-phenylindole (DAPI) staining was shown in plan A3 and B3. C: Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess the expression of spermatogonial cells genes, specifically OCT4, VASA, PLZF, and INTG $\alpha$ 6, during the entire culture. All samples expressed specific spermatogonial genes.

**Table-1:** Name and properties of designed primers used for RT-PCR.

Gene	The sequence of Primers (5'-3')	Annealing temperature(C0)	Size(BP)
4-Oct	F: GTTCTTCATCTAGGAAGG R: CAAGAGCATCTGAACCTCAC	60	100
PLZF	F: GGTCGAGCTTCTGATAACG R: CCTGTATGTGAGCCGAGGT	55	396
VASA	F:TACTTGCTGGACGAGATTCTG R: ATCCATCAGTCTCCAGGAG	62	320
INTG $\alpha$ 6	F: TCATGGATCTGCAAATGGAA R: GCGGGTTAGCAGTATATCA	52	300
$\beta$ - actin	F: CAAGATCATCTCCTCCTG 3' R:ATCCACATCTGCTGGAAGG 3'	60	90

**Table-2:** Comparison of the number and diameter of the colonies in three groups, separately.

Groups colonies	Diameters of colonies		Number of colonies
	After 7 weeks	After 7 weeks	
Control	81.6 $\pm$ 12	3.5 $\pm$ 1	
Growth factors(GF)	165.2 $\pm$ 32.5*	11 $\pm$ 2.2*	
Growth factors plus collagen(GF+Col)	276.6 $\pm$ 45.5*†	18 $\pm$ 2.6*†	

\*Significant difference between GF and GF+Col groups with the control group (p < 0.05).

†Significant difference between GF and GF+Col groups (p < 0.05).

Testicular cells consisted of spermatogenic and somatic cells, as well as FACS, and were used to remove testicular somatic cells and to enrich the suspension with SSCs. After two-step enzymatic digestion, the cell suspension contained cells with different size and morphology. After FACS-sorted for CD49f selection, the enriched fraction of fresh cell was homogenous, containing cell with similar sizes and shapes (Figure-1C).

The cells were cultured for 7 weeks. After 2-7 weeks, the cluster and colonies appeared in different groups. Earlier colonies appeared in growth factor plus collagen group after 8 days in culture, in the growth factor group they appeared on day 12 and in the control group on day 19. Our results indicated that number (n) of the colonies and diameters (d) in the growth factor plus collagen groups were more than other groups. Besides number of the colonies and diameters in growth factor group were significantly better than control group (p < 0.05) (Figure-2; Table-2).

After fifth passage, the colonies were divided into two groups. One was fixed for immunocytochemical characterisation and the other was prepared for RT-PCR analysis. Immunocytochemical analysis revealed the

expression of OCT4 in the colonies among different study groups (Figure-3).

Total RNA was extracted at the end of week 7, and RT-PCR was performed to analyse the expression of specific spermatogonial cells markers, including PLZF, OCT4, VASA and INTG $\alpha$ 6. All samples expressed specific spermatogonial genes (Figure-3C).

## Discussion

In vitro proliferation and enrichment of the SSCs is important, and colonisation of these cells provides the valuable source for next studies such as genetic manipulation, freezing and infertility treatment as well. In our culture, the addition of EGF, LIF and GDNF on collagen-coated dishes increased the number of SSCs by self-renewal in vitro. EGF expresses in the testes after birth, and has numerous effects on the differentiation and proliferation of Sertoli cells, Leydig cells and peritubular cells.

GDNF secretion by Sertoli cells is controlled by the follicle stimulating hormone (FSH). SSCs express specific receptor of GDNF known as C-RET and GFR  $\alpha$ 1. Apparently, the GDNF acts through several different paths for self-renewal and the survival of SSCs.

Also, LIF has an important role in the regulation of Sertoli cells and germ cells function. The mentioned factors play an important role in niche establishment through maintaining the balance between self-renewal and differentiation.<sup>22</sup> Many studies in humans<sup>7,8,11</sup> have showed the beneficial effects of mentioned growth factors on SSCs proliferation. Generally, stem cells reside within a special microenvironment or 'niche', which provides factors that regulate the proliferation and differentiation of the stem cell population.<sup>23</sup> SSCs expand in the presence of feeder layer cells such as mouse embryonic fibroblast (MEF) cells, Sandos Inbred Mouse (SIM) mouse embryo-derived Thioguanine and Ouabain resistant cells (STO cells) and human embryonic cell-derived fibroblast-like cells (hEFs).<sup>24</sup> However, use of xenogeneic or allogeneic feeder cells for culturing human SSCs is associated with risks such as pathogen transmission and viral infection. Moreover, availability of hEFs from aborted fetuses is relatively low, and Sertoli cells cannot support the culture of undifferentiated SSCs equally well. All of these factors limit further application of human SSCs for therapy. Therefore, it is necessary to develop an improved culture system that can support the growth of human SSCs. Sertoli cells are the main cells in the niche. Based on previous studies,<sup>11</sup> we decided to use Sertoli cells as a feeder layer in addition to growth factors in vitro to create a testis-like microenvironment to better support the Sertoli cells and SSCs.

In a recent study Koruji et al. in 2012 studied the effects of laminin and growth factors on the proliferation of human SSCs obtained from NOA patients. Results showed that the addition of EGF, bFGF, LIF and GDNF on laminin-coated dish significantly increases cluster formation compared to the control group.<sup>11</sup>

Collagens are ubiquitous structural proteins found in ECM of mammalian tissues, including the testis. Type IV collagen, a network-forming collagen type,<sup>14</sup> and laminins are the most abundant building blocks of the basement membrane.<sup>15-17</sup> Collagen can take part in signal transduction via transmembrane receptors, such as integrins.<sup>16,18</sup> It was shown that NC1 fragments of collagen take part in the regulation of adhesion, proliferation and apoptosis in various cell types via their interactions with integrins, such as  $\alpha$ 6 $\beta$ 1 and  $\alpha$ v $\beta$ 3 integrins. These findings have clearly illustrated the signalling function of collagen.<sup>19</sup> In the present study, we isolated CD49f ( $\alpha$ 6 integrin) cells using LIF, EGF, GDNF and collagen-coated dish to improve the survival and proliferation of cells in vitro. Results indicated that number of the colonies and their diameters in the growth factor-plus-collagen groups were more than other groups and differences were significant. Also, number of the colonies and their diameters in growth factor group were better than the control group and the differences were significant. In the control group, Sertoli cells were separated gradually after 4 weeks.

Despite significant increase in colony diameter, number of colony during various days of culture in each group did not show significant difference that could represent harmony and synchronisation stimulation of human SSCs during culture.

Studies have been performed in relation to the effect of different culture systems on gene expression in the SSCs and the nature of the cells evaluated based on the amount of gene expression.<sup>7,9,11</sup> In the present study, spermatogonial-derived colonies showed OCT4 expression. This finding is in agreement with previous investigators who demonstrated OCT4 expression in colony cells.<sup>7,9,11</sup>

Based on various studies, maintaining appropriate conditions for the sustainability of the presence of human SSCs during the culture is difficult. This issue could be due to incompetent culture condition.

In our study, analysis of the expression of specific markers of sexual cells represented the presence of human SSCs in different groups. For confirmation of the presence of SSCs during culture, RT-PCR was performed to investigate the

expression of specific spermatogonial markers (PLZF, OCT4, VASA, and INTG $\alpha$ 6). Our results related to these markers' expression were similar to previous reports.<sup>8,11</sup>

## Conclusion

In vitro proliferation and enrichment of the human SSCs in our culture system enabled the colonization of these cells, and it may provide a valuable source for future studies.

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