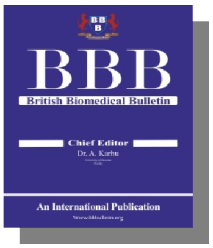
Journal Home Page www.bbbulletin.org

BRITISH BIOMEDICAL BULLETIN



Original

Isolation of Native Goat Spermatogonial Cells and Co-culture with Sertoli Cells

Masomeh Mohebbi[1] and Gholamali Moghaddam*[2]

[1]*Graduated student, Department of Animal Science, Faculty of Agriculture, University of Tabriz, Iran*
[2]*Department of Animal Science, Faculty of Agriculture, University of Tabriz, Iran*

A R T I C L E I N F O

Received 12 Oct. 2014
Received in revised form 25 Oct. 2014
Accepted 27 Oct. 2014

Keywords:
Co-culture,
Isolation,
Goat,
Sertoli cells,
Spermatogonial cells.

Corresponding author: Department of Animal Science, Faculty of Agriculture, University of Tabriz, Iran.
E-mail address:
ghmoghaddam@tabrizu.ac.ir

A B S T R A C T

Isolating and co-culturing of native goat spermatogonial cells with Sertoli cells was the aim of this study. Stem cells are very important in medical, biological, veterinary and animal researches. There are different types of stem cells but through of them, spermatogonial cells are special because they are only cells in male body that can transport genetic information to next generation. Sertoli cells are a kind of cells that support spermatogonial cells. For producing of recombinant proteins, goat is one of the suitable domestic animals. In this study two month age native male kids were used to isolate stem cells. Dulbecco's Modified Eagle's Medium (DMEM) was enriched with 10% Fetal Bovine Serum (FBS). The viability of cells was evaluated after isolation and co-culture steps. In this paper morphological differentiation and immunocytochemistry tests were used to identify of cells. Anti-vimentin and anti-Oct-4 immunocyto-chemical staining method identified Sertoli cells and SSCs, respectively. The number of spermatogonial colony and their area were evaluated by scaled lens. Finally, native goat spermatogonia were isolated and cultured using co-culture method with Sertoli cells.

Introduction

Development[1] of new research tools allow the genetic improvement and production of transgenic animals and saving of in dangerous species.

Stem cells are the cells with self renewal and differentiation capacity; they can transmit genetic information to next generation[1,2]. Spermatogenesis is a process that supported by spermatogonial stem cells (SSCs). There are rare spermatogonial stem cells (SSCs) in testis that support spermatogenesis. 0.02‑0.03% of total germ cells, 1.25% of all spermatogonia, and 10.6% of undifferentiated spermatogonia in the rodent testis are SSCs, so amounting to 35,000 SSCs per mouse testis[3,4].

Sertoli enrico described the Sertoli cell (SC) in 1865 first time. These cells have close structural relationship with the spermatogenic cells in the seminiferous tubules, so scientist's attentions focus them[5]. Defined number of germ cells and spermatogonia were supported by each sertoli cells[6]. Sertoli cells are essential for testis formation and spermatogenesis. These cells support the germ cells to change to spermatozoa via direct contact and by controlling the microenvironment in the seminiferous tubules[5].

A simple way to achieve great number of spermatogonial cells are that immature animals are used. In adult animals using of cryptorchid species or animals with deficiency of vitamin A is essential to get high number of stem cells. There is different types of germ cells in adult animals so isolating of spermatogonial cell from adult animal is difficult vs. immature animals[7,8].

As SSCs require a niche provided by Sertoli cells *in vivo*, probably this microenvironment can be reproduced *in vitro*. We can hold this as valid for the culture system. Accordingly, a good system would be composed of a starting population of a highly purified SSC suspension with autogenous Sertoli cells. These epithelial cells provide self-renewal proteins to SSCs in their niches in the testis[9]. The first step of working on spermatogonial cell is the isolation and culture of them. There are different experiments that used co-culture system for Sertoli cells and spermatogonial cells[10-14]. Co-culturing of spermatogonial cells with Sertoli cells showed a significant increase in the number and diameter of the colonies compared with the treated growth factors and the control group[15].

Culture media with animal serum is necessary for cell growth and for the stimulation of proliferation ("mitogenic effect"). The sera that used most widely are from adult or newborn animals or of fetal bovine origin. Whole animal serum is an extremely complex mixture of a large number of constituents, low and high molecular weight biomolecules with different physiologically balanced growth-promoting and growth-inhibiting activities as well as fetal bovine serum (FBS)[16].

Spermatogonial stem cells can't expand in a culture medium without serum and feeder layer more than one week[17]. The scientists have tried for years to perform suitable condition for living and proliferation of these cells but they can't have more progress in this aspect because of lack of knowledge of nutritional require of these cells. Adding of serum in medium increases viability rate of spermatogonial cells. In free serum condition 20%-30% of cell number has been decreased[18], our research group used 10% FBS and co-culture method.

For producing of recombinant proteins, goat is one of the suitable domestic animals[19]. Therefore, there is an interest in the application of SSCs as an alternative approach to create transgenic animals. The goat is economically a very important animal but there are a few data about its reproduction system. The seminiferous epithelium cycle period duration in goat is

10.6 days. Total period of spermatogenesis is 4.5 epithelium cycle in goat. (10.6*4.5= 47.7) so the length of spermatogenesis is 47.7 days[20].

At the onset of culture, spermatogonia is round cells with a spherical nucleus and one to three dense, spherical nucleoli of approximately 1–3 mm in diameter occupying a central or slightly eccentric position in the nuclei; a high nucleus: cytoplasm ratio; and many cytoplasmic inclusions, mostly concentrated at one side of the cell. After a few days, the somatic cells constitute a confluent monolayer[21].

The identity of the cells confirm through analysis of immunocytochemistry against Oct-4 and vimentin. Vimentin is a cytoskeletal protein. It usually found in epithelial cells and it is a molecular marker of Sertoli cells detect in the feeder monolayer cells. The great majority of the cells in the monolayer are positive for the Sertoli cell marker vimentin[15].

For confirmation of the presence of spermatogonial cells, in addition to morphology, Oct-4 trace in the colony cells. SSCs show Oct-4 expression[22].

The aim of this study was to isolate and co-culture goat spermatogonial cells with Sertoli cells in a medium that enrich with 10% FBS. Spermatogonial stem cells and Sertoli cells were identified by immunochemistry test and through of this study the morphology of spermatogonial stem cells and Sertoli cells was shown.

Materials and methods

Animals and sampling

Two month age native kids at the Khalatposhan Research Institute of Tabriz University were used. 10 replicates were performed. TESE technique was performed for getting samples. Samples were taken in DMEM with 10% FBS and were transferred to laboratory in two hours.

Cell collection

For achieving of cells enzymatic digestion protocol with some modification was used[12]. Briefly, testis pieces that obtained with TESE technique were mechanically minced and floated in DMEM. DMEM contained 1 mg/ml collagenase, 1 mg/ml Trypsin, 1 mg/ml hyaluronidase type II and 5 µg/ml DNase I. These substances incubated at 37°C for 60 min. After this step, the suspension was centrifuged at 1400g for 2min. Then by removing of supernatant, semineferous tubles were incubated with secondary step enzymes that including 1mg/ml collagenase, 1mg/ml hyaluronidase type II and 5µg/ml DNase. This step was taking 15min in 37°C incubator.

In the second step, spermatogonial and Sertoli cells were achieved as cellular suspension. Finally spermatogonial cells were co-cultured with Sertoli cells at incubation temperature of 37°C, atmosphere of 5% CO_2 in a humid chamber were used. Cell growth and colony formation ability were assayed in 4, 8 and 12 days after co-culture by invert microscope. Medium cells were changed in 4, 8 and 12 days after co-culture and before assaying of number and area of colonies.

The medium contained DMEM high glucose with 10% FBS, 2 *mM* L-Glutamin, 100 µ*g/ml* streptomycin, 100 *IU/ml* penicillin.

Colony counting and viability rate

The number of colony were count by inverted microscope in 4, 8, 12 days after co-culturing.

For differentiating of viable and nonviable cells, Trypan Blue was used. Cells are very selectable when it comes to allowing or baring compounds to pass through the cell membrane[23].

After 12 days co-culture, the viability of the cells was determined using

0.4% trypan blue and light microscope (Olympus, Tokyo, Japan).

Spermatogonial and sertoli cell identification

For identification of Sertoli cells anti-vimentin were used[14]. Briefly, anti-vimentin diluted in TBS/BSA (5µg/ml and 2µg/ml, respectively) was applied over slides for 60min at 25°C. Fluorescein Isothiocyanate (FITC)-conjugated sheep anti-mouse Ig was diluted in TBS/BSA in a ratio of 1:50 and incubation was further continued for 45min at 25°C. Following once washing with TBS/BSA, slide was exposed to DAPI for 5min. It was then washed and mounted in PBS-glycerol 90% and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Oct-4 was described as a marker for undifferentiated cells. Anti-Oct4 immuno-cytochemically stained obtained colonies of Spermatogonial cells with (conjugated with FITC). Briefly, anti-Oct4 (Abcam) diluted in TBS/BSA, was applied over slides for 60min at 25°C. After washing, FITC conjugated donkey polyclonal secondary antibody to Goat Ig G was added and incubation was further continued for 45min at 25°C. After washing with TBS/BSA, it was mounted in PBS-glycerol 90%, and examined under a fluorescence microscope (Olympus, Tokyo, Japan)[13].

Statistical analysis

Results are presented as the mean 6 SEM. Student t-test was used for statistical analysis. Differences were evaluated to be significant at $P<0.05$.

Results

SSCs are present in the basement membrane of the seminiferous tubules, in specific niches in close contact with supporting Sertoli cells and surrounded. In laboratory after first enzymatic digestion, the interstitial tissue and cells were removed and after incubation of seminiferous tubules, cell suspensions were centrifuged to achieving of cells. The result of enzymatic digestion was achieving of spermatogonial cells and Sertoli cell.

Isolation and characterization of spermatogonial cells and Sertoli cells: The Sertoli cell population created a monolayer of cells 3 days after co-culturing (Fig. 1). The Sertoli cells were evidenced by their morphology.

Cell growth and colony formation ability were assayed in 4, 8 and 12 days after co- culture.

Trypan blue is a stain that penetrates in dead cells. The blue stain is easily visible, and cells can be counted using a light microscope. The viability of spermatogonial cells after 12 days co-culture in DMEM with 10% FBS was > 93%.

Identification of cells: As it obvious in Fig. 4, the spermatogonial cells are round cells and Sertoli cells are triangle cells. Immunochemistry showed that vimentin was detected in feeder monolayer cells (Fig. 4A). As depicted in Fig. 4, vimentin localized in Sertoli cells indicating expression of this intermediate filament protein in these cells. Spermatogonial cells were expressed Oct-4 by immunocytochemistry staining (Fig. 4B).

The nuclei of both Sertoli and spermatogonial cells were immunostained with DAPI (Fig. 4A, B).

As a result of our study, spermatogonial cells were round with a size about 14 µm. The colony of spermatogonial cells was observed in this Figure. The colonies appeared four days after co-culturing step (Fig. 5).

Discussion

Initial attempts at isolating SSCs started with the isolation of “type A” spermatogonia (containing SSCs) by enzymatic digestion of testicular tissue[24,25].

At present study immature animals were used and a great population of these cells was got[7,8].

SSCs require a niche provided by Sertoli cells *in vivo*, probably this microenvironment can be reproduced *in vitro*[24].

This study demonstrates that highly spermatogonia can be obtained from the testes of goats. Co-culturing with Sertoli cell is one way that leads us to achieve a highly number of colonies of spermatogonial stem cell. Scientists co-cultured human spermatogonial stem cells with Sertoli cells[26]. kruji *et al* studied efficiency of adult mouse spermatogonial stem cell colony formation under several culture conditions and used co-culture method and had succeeded. Izadyar *et al* performed an experiment on bovine testis and culture type A spermatogonia with Sertoli cells[12]. Co-culturing of goat spermatogonial stem cell with Sertoli cells in 2012 using different serum concentrations was performed by Bahadorani *et al*. Their purpose was to investigate the effect of serum supplementing on short-term culture. They used Crude testicular cells[10] but in our research, Testicular Sperm Extraction (TESE) technique was run and a piece of seminiferous tubule was brought in lab and these cells were co-cultured with Sertoli cells after isolation.

Culturing of suspension isolated cells were used in this study. There are two culture systems in laboratory for spermatogenesis, first of them is culturing of testis organ and second is culturing of suspension isolated cells. However the first way is interesting but because of complex condition of testis structure and its physiological condition to prepare of nutritional condition that effect on special cells is difficult, the second way usually use[24].

The advancement of amphibian spermatocytes through meiosis and some aspects of the maturation of spermatids require the presence of Sertoli cells in the co-culture. A similar reliance on Sertoli cells in the mammalian testis can be postulated[5].

In mouse Sertoli cells formed a confluent layer and after fortten days, the collected cells were co-cultured on the top of Sertoli cells so Sertoli cells prepared with more than 95% purity[15].

By analyzing of immunocyto-chemistry against Oct-4 and vimentin the identity of the cells was confirmed. Vimentin is a cytoskeletal protein. It usually found in epithelial cells. Vimentin is in the perinuclear region of the cells[28,29], It is a marker for day 14 postnatal Sertoli cells[27]. Moreover vimentin which is a molecular marker of Sertoli cells was detected in the feeder monolayer cells.

In addition to morphology, Oct-4 for presence of spermatogonial cells was used. SSCs-derived colonies showed Oct-4 expression. This finding is in agreement with that reported by previous investigators[15,22,30] who demonstrated Oct-4 expression in the spermatogonial cells but it is in disagreement with that reported by Richards *et al*[31].

In conclusion, the results of present investigation demonstrated that isolation and co-culture of SSC with Sertoli cells in goats is possible.

References

1. De Rooij D.G, Russell L. All you wanted to know about spermatogonia but were afraid to ask. *J. Androl*. 2000; 21, 776–798.
2. Brinster R. Germline stem cell transplantation and transgenesis. *Science* 2002; 296, 2174–2176.
3. Tegelenbosch RA, de Rooij D.G. A quantitative study of spermatogonial multiplication and stem cell renewal in the

C3H/101 F1 hybrid mouse. *Mutat Res* 1993; 290:193–200.
4. Meistrich ML, van Beek MEAB. Spermatogonial stem cells. In: Desjardins CC, Ewing LL, editors. Cell and molecular biology of the testis. New York: Oxford University Press1993; 266–95.
5. Griswold M.D. The central role of Sertoli cells in spermatogenesis. *Seminars in cell & developmental biology*1998; 9:411-416.
6. Meachem S, Viktoria von Schönfeldt V, Schlatt S. Spermatogonia stem cells with a great perspective. *Germany Reproduction*. 2001; 121:825–834.
7. Kubota, H.; Avarbock, M. R.; Brinster, R. L. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc. Natl. Acad. Sci*. U. S. A. 2004b; 101: 16489‑16494.
8. Van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, *et al*. Isolation of the synchronized A spermatogonia from adult vitamin Adeficient rat testes. *Biol Reprod* 1996; 55: 439-444.
9. Meng X, Lindahl M, Hyvonen M. E, Parvinen M, de Rooij, D.G., Hess M. W, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel J. G, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287: 1489–1493; 2000. doi:10.1126/science. 287.5457.1489-1493.
10. Bahadorani m, Hosseini SM, Abedi P, Hajian M, Hosseini SE, Vahdati A, Baharvand M, Nasr-Esfahani MH. Short-term in-vitro culture of goat enriched spermatogonial stem cells using different serum concentrations. *J Assist Reprod Genet* 2012; 29:39–46.
11. Nakanishi Y, Shiratuchi A. Phagocytic Removal of Apoptotic Spermatogenic Cells by Sertoli Cells. Mechanisms and Consequences Biol. *Pharm Bull* 2004; 27(1):13-16.
12. Izadyar F, Spierenberg G. T, Creemers L. B, den Ouden K, de Rooij D. G. Isolation and purification of type A spermatogonia from the bovine testis, *Reproduction* 2002;124:85-94.
13. Qasemi-Panahi B, Tajik P, Movahedin M, Moghaddam G.H, Barzgar Y, Heidari-Vala H. Differentiation of Bovine Spermatogonial Stem Cells into Osteoblasts. *Avicenna J Med Biotech* 2011; 3(3): 149-153.
14. Tajik P, Barin A, Movahedin M, Zarnani A.H, Hadavi R, Moghaddam G.H, Shoja J, Jeddi-Tehrani M, Ashrafi-Helan J, Heidari-Vala H, Torkabadi E, Qasemi-Panahi B. Nestin, a neuroectodermal stem cell marker, is expressed by bovine Sertoli cells Comp Clin Pathol 2010.
15. Koruji M, Movahedin M, Mowla S. J , Gourabi H, Arfaee A. J. Efficiency of adult mouse spermatogonial stem cell colony formation under several culture conditions *In vitro*. *Cell. Dev. Biol Animal* 2009; 45:281-289.
16. Gstraunthaler G. Alternatives to the Use of Fetal Bovine Serum: Serum-free Cell Culture. *ALTEX* 2003;20, 4/03: 275-281
17. Creemers L.B, Ouden K, Van Pelt A, De rooij D.G. Maintenance of adult mouse type A spermatogonia *in vitro*: influence of serum and growth factors and comparison with prepubertal spermatogonial cell culture *Reproduction* 2002; 124:791–799.
18. Morena A.R, Boitani C, Pesce M, Oe Felici M, Stefanini M. Isolation of highly purified type A speramtogonia from prepubertal rat testis. *J Androl* 1996; 17:708-717.
19. Meade HM, Echelard Y, Ziomek CA, Young MW, Harvey M, Cole ES, *et al*. Expression of recombinant proteins in the milk of transgenic animals. In: Fernandez JM, Hoeffler JP, editors. Gene expression systems: using nature for the art of expression. San Diego: *Academic Press* 1998; 399–427.
20. França LR, Becker-Silva SC, Chiarini-Garcia H. The length of the cycle of seminiferous epithelium in goats (Capra hircus). *Tissue Cell*. 1999; 31:274-280.
21. Izadyar F, den Ouden k, Creemers L, Posthuma G, Parvinen M, de Rooij D. Proliferation and Differentiation of Bovine Type A Spermatogonia During Long-Term Culture. *Biology of reproduction* 2003; 68, 272–281.
22. Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors

affect fate determination of mouse spermatogonial stem cells. *Biol Reprod* 2004a; 71: 722-731.
23. Suganuma M, Sayama Y, Nakamura M. Trypan blue staining capacities of the culture cells, the mechanism of staining. *Rinsho Byori* 1989; 37(12):1361-6.
24. Aponte PM, van Bragt MP, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. *APMIS*. 2005; 113: 727-742.
25. Clermont Y. Spermatogenesis in man. A study of the spermatogonial population. *Fertil Steril*.1966; 17: 705-721.
26. Liu S, Tang Z, Xiong T, Tang W. Isolation and characterization of human spermatogonial stem cells. *Reprod Biol Endocrinol* 2011; 9:141. doi:10.1186/1477-7827-9-141.
27. Scarpino S, Morena A. R, Petersen Cryopreservation, Froysa B, Soder O, Boitani. Cryopreservation A rapid method of Sertoli cell isolation by DSA lectin, allowing mitotic analyses. *Mol. Cell Endocrinol* 1998; 146: 121–127.
28. Oke B. O, Suarez-Quian C. A. Localization of secretory, membraneassociated and cytoskeletal proteins in rat testis using an improved immunocytochemical protocol that employs polyester wax. *Biol. Reprod*. 1993; 48:621–631.
29. Mori C, Nakamura N, Dix D. J, Fujioka M, Nakagawa S, Shiota K, Eddy E. M. Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp 70-2 knockout mice. *Dev. Dyn*. 1997; 208:125-136.
30. Shi Y. Q, Wang Q. Z, Liao S. Y, Zhang Y, Liu Y. X, Han C. S. *In vitro* propagation of spermatogonial stem cells from KM mice. *Front Biosci* 2006; 11: 2614–2622.
31. Richards A. J, Enders G. C, Resnick J. L. Differentiation of murinepremigratory primordial germ cells in culture. *Biol. Reprod* 1999; 61: 1146–1151.

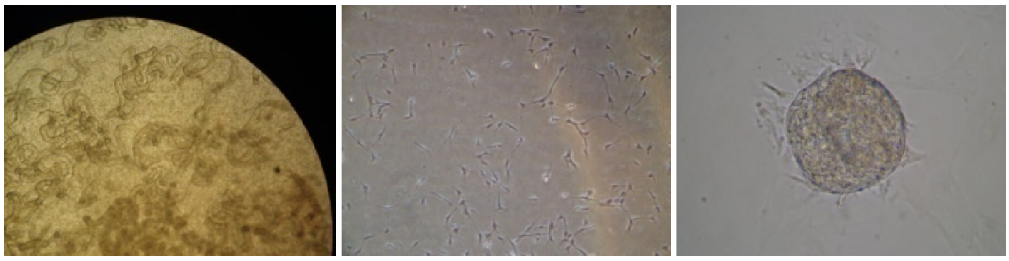


Figure 1. (A) semineferous tubules were shown after first enzymatic digestion (light microscope *40). (B) Sertoli cells created a monolayer cells (inverted microscope *40). (C) A colony of spermatogonia around with Sertoli cells (inverted microscope *100)

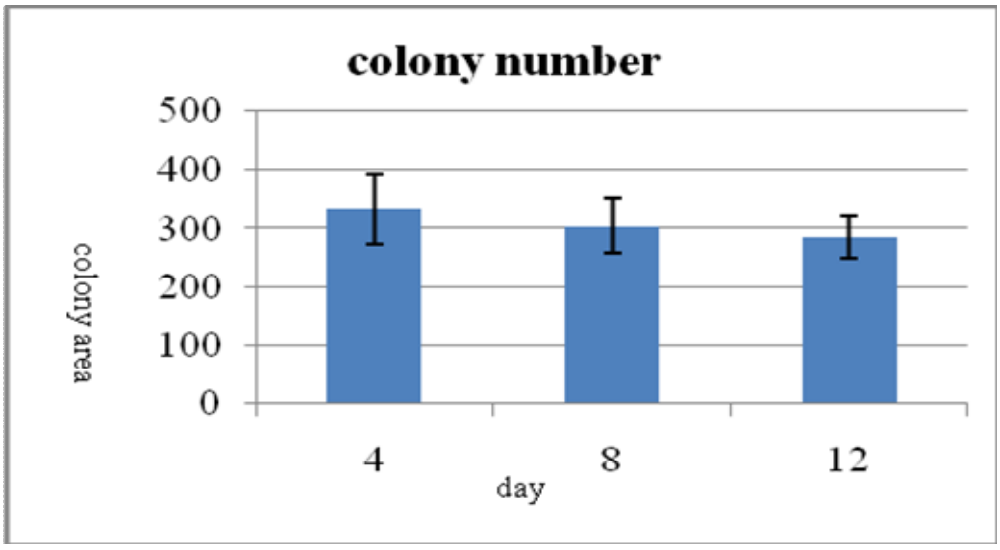


Figure 2. Relationship between colony number and time. There is no significant difference between colony number and different days of culturing

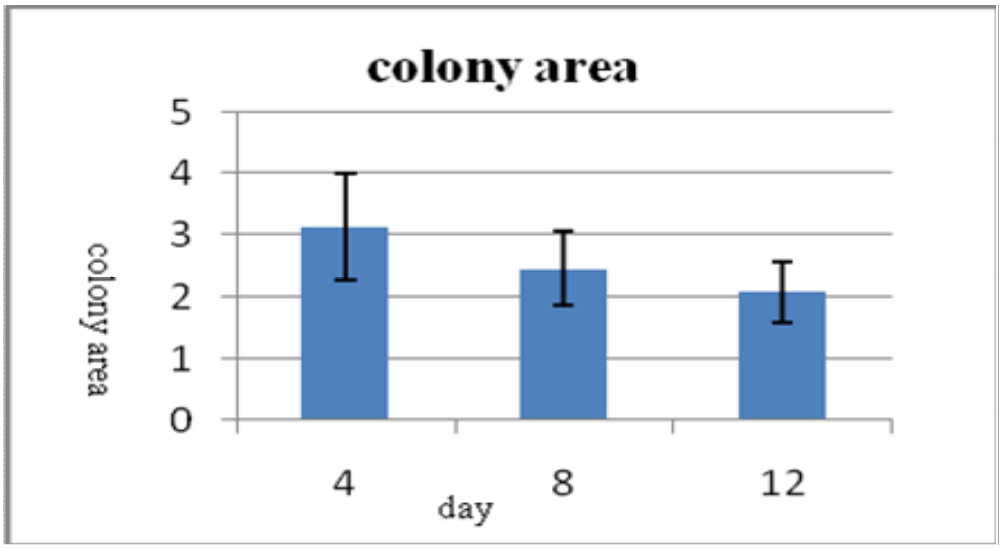


Figure 3. Relationship between colony number and time. There is no significant difference between colony area and different days of culturing

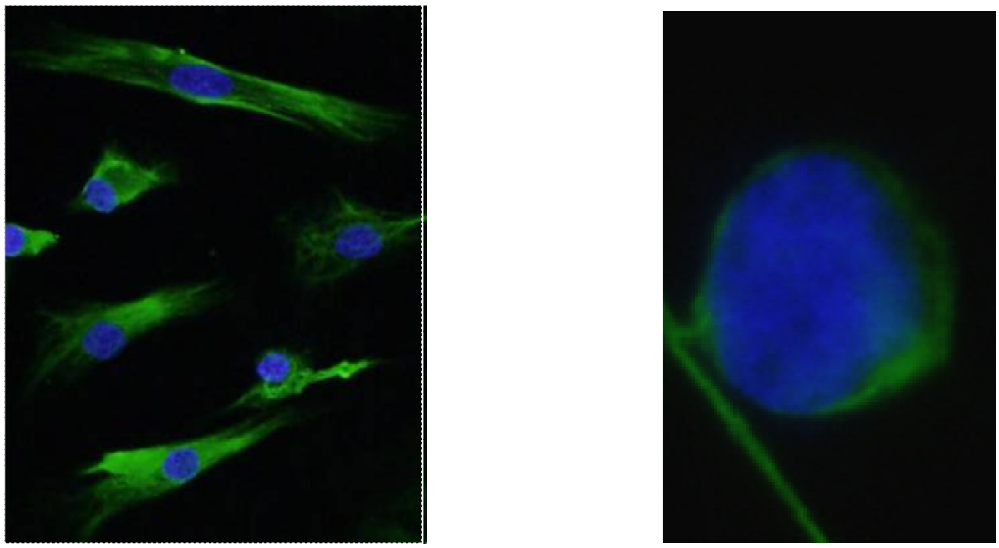


Figure 4. Immonuchemistry tests. (A) Sertoli cells (*200) (B) spermatogonial cells (*400). The voulume of nucleus in spermatogonial cells are more than nucleus voulume of Sertoli cells

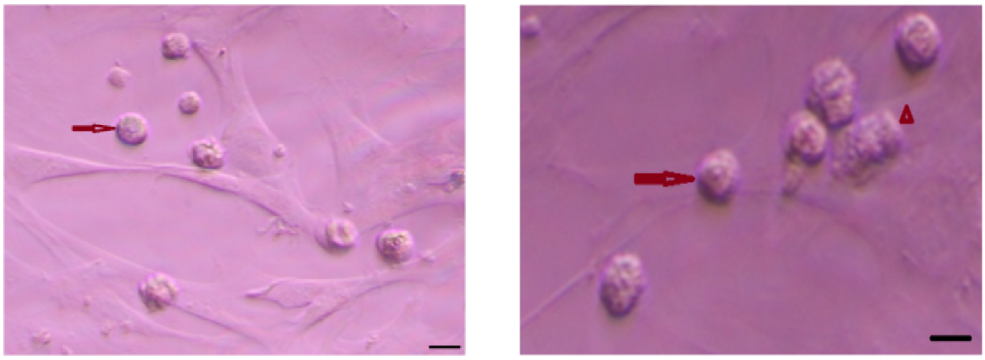


Figure 5. Spermatogonia (red arrow), Colony of spermatogonia (arrowhead), Bar= 15µm