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Fetal bovine serum (FBS) enhances proliferation and colonization of caprine spermatogonial stem cells

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ABSTRACT

Enrichment of cell suspension with germ cells prior to injection into recipient seminiferous tubules is of importance in spermatogonial stem cells (SSCs) transplantation. Fetal bovine serum (FBS) is the most widely used growth supplement for cell cultures, primarily because of its high levels of growth stimulatory factors and low levels of growth inhibitory factors. This study was undertaken to investigate the effect of serum concentration on colony formation and development of different types of SSC colonies with respect to passage number. Cells were isolated from pre-pubertal buck testes by two step enzymatic digestion method. The filtered cells were enriched by differential adherence selection method. Cells were then randomly divided into 8 groups, depending on concentration of FBS in culture medium ranging from 0% to 35%. In experiment 1, effect of different concentrations of FBS on total number pSSCs with reference to differential plating was observed while in experiment 2, effect of different concentrations of FBS on types of pSSC colonies with respect to passage number was observed. No colony formation was observed in control group (0% FBS) while significantly higher number of single, paired, cluster and rosette colonies observed were with 20% FBS group in differential 2 (D2) as compared to other groups. Alkaline phosphatase staining and immunocytochemistry staining (PGP9.5 and OCT4) were positive in SSCs colonies. The growth rate of the culture was significantly and consistently higher with 20% FBS.

Keywords: *Capra hircus,* Differential plating, Fetal bovine serum, Pre pubertal testis, Spermatogonial stem cells

Spermatogonial stem cells (SSCs), the germ stem cells of the seminiferous epithelium in the testis are the founder cells of a sperm producing stem cell system called spermatogenesis. Spermatogenesis is highly coordinated and complex process, consisting of three distinct phases (Takashima *et al.* 2018). SSCs are the only adult stem cells capable of transmitting the genome of a given species from one generation to the next, while at the same time having the capacity to convert into pluripotent stem cells (Kanatsu-Shinohara *et al.* 2008). As SSCs are rare in the testis, presumably 1 in 3000–4000 cells in adult mouse testis (Adetunji *et al.* 2018), several approaches to enrich stem cells have been attempted. For high yield cell culture, one needs a medium that supplies the necessary nutrients and growth factors. The chemical composition of the medium is very complex and many components of culture medium can affect the rate at which cells proliferates, out of which serum represents one of the best documented modulator of cell proliferation.

Fetal bovine serum (FBS) is the liquid fraction of clotted blood from fetal calves, depleted of cells, fibrin and clotting factors, but containing a large number of nutritional and macromolecular factors essential for cell growth. Bovine serum albumin is the major component of FBS. Growth factors in FBS are essential for the maintenance and growth of cultured cells (VanDer Valk *et al.* 2018). FBS also contains a variety of small molecules like amino acids, sugars, lipids, and hormones. One of the primary uses of FBS is in eukaryotic cell culture, where it provides many essential nutrients and growth factors that facilitate cell survival and proliferation. Taking advantage of the beneficial effects of serum, the pattern of serum supplementation was studied. In the present study, different types of putative SSC (pSSC) colonies using different concentrations of FBS without growth factors are reported.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical (St Louis, MO, USA), unless otherwise mentioned. Plasticware was purchased from Tarsons (Rosklide, Denmark) and the nylon mesh filters from Millipore (Bedford, MA, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (USA) and antibodies were purchased from Abcam (US).

Isolation and enrichment of caprine spermatogonial stem cells: Goat testes were collected from pre-pubertal bucks (3 to 6 months of age) immediately after the slaughter from a local abattoir. Testes were transported to the laboratory within 1 h in normal saline solution fortified with

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streptomycin (500 µg/ml) and penicillin (100 IU/ml) where they were again washed 3 to 4 times with Dulbecco's phosphate buffered saline (DPBS). Subsequently, tunica albuginea was removed with a sharp surgical blade and 4–5 g of the exposed seminiferous tubules were isolated and minced in DPBS with antibiotics (streptomycin (500 µg/ml) and penicillin (100 IU/ml)). The minced tissue was subjected to enzymatic digestion, as per the previously described protocol (Heidari *et al.* 2012) with some minor modifications. Briefly, minced tissue were suspended in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase type II, 5 µg/ml DNase I and 1 mg/ml trypsin at 37ºC in a shaker incubator for 45 min. Supernatant after washing at 1,000 rpm for 5 min with DMEM media containing antibiotics was discarded for removal of interstitial cells. Seminiferous cord fragments were then given second digestion with trypsin, collagenase, hyaluronidase and DNase with same concentration for 30 min as described above. The supernatant after centrifugation, which was presumed to contain SSCs, sertoli cells, myeloid cells and other contaminating cells of the seminiferous tubular tissue, was filtered through 80 µm and then 60 µm nylon mesh filters to enrich the SSC population.

The pSSC population isolated in DMEM medium with gentamycin sulphate (50 µg/mL), antimitotic solution antibiotics (10 µl/mL), non-essential amino acids (1%) and L-Glutamine (1%) were randomly divided into 8 groups, viz. *Group A* (*Control*): pSSCs were cultured in DMEM culture media containing 0% FBS; *Group B:* pSSCs were cultured in DMEM culture media containing 5% FBS; *Group C:* pSSCs were cultured in DMEM culture media containing 10% FBS; *Group D:* pSSCs were cultured in DMEM culture media containing 15% FBS; *Group E:* pSSCs were cultured in DMEM culture media containing 20% FBS; *Group F:* pSSCs were cultured in DMEM culture media containing 25% FBS; *Group G:* pSSCs were cultured in DMEM culture media containing 30% FBS; *Group H:* pSSCs were cultured in DMEM culture media containing 35% FBS.

Experiment 1 (*Effect of different concentration of FBS on types of pSSCs*)*:* On day 0, freshly isolated cells were cultured into 24 well plates for 24 h until the fully attachment of somatic cells to the bottom of the plate in culture medium (D1) was observed. In differential (D2), after adherence of somatic cells, the floating cells were aspirated and gently transferred in a fresh well with culture medium and then incubated at 37°C in humidified atmosphere with 5% CO_2. Media was replaced every alternate day. This method is called differential adherence technique and is done for enriching the stem cell population. Cells isolated were cultured in DMEM with different FBS concentrations, for 20 days. The number of colonies were counted on 12th day.

Experiment 2 (Effect of different concentration of FBS on total number of pSSC colonies with respect to passage number): After 60–70% confluency, cells were detached from 24 well plate by using 0.25% trypsin/EDTA for 1–2 min at 37°C and seeded into a new plate for subsequent passages. The cells were cultured up to Passage 11 in this study. Number and types of colonies were evaluated on 3rd day before every passage.

Characterization of spermatogonial stem cells: Alkaline phosphatase staining was performed using a commercially available kit (catalogue no. 86C; Sigma Chemical), as per the kit instructions. The medium was removed from the cultures, and were washed thrice with DPBS. This was followed by fixation with citrate–acetone–formaldehyde fixative solution for 1 min and three washings with deionised water. After that alkaline dye was added and the cultures were incubated for 15 min at room temperature. The dishes were rinsed three times with deionised water and counterstained with neutral red stain for 1–2 min. The colonies were washed several times to remove the extra neutral red stain. The red coloured colonies were considered positive for alkaline phosphatase activity and were presumed to be SSCs.

The expression of OCT 4 and PGP9.5 in pSSC colonies was examined by immunofluorescence staining. The colonies were fixed in 4% paraformaldehyde in DPBS for 30 min and permeabilised by treatment with 0.5% Triton X-100 in DPBS for 30 min. The cells were incubated with blocking solution (2% BSA) for 30 min and then with primary antibodies mouse monoclonal PGP9.5 and OCT4 (1: 10) for 1 h at room temperature followed by washing with DPBS. FITC labeled secondary antibodies were added dilution of 1: 100 to 1: 200 (goat anti-rabbit IgG) followed by incubation for 2 h at RT in dark. SSCs were then examined in a fluorescent microscope. After counterstain with DAPI (0.1 µg/mL) for 1 min, cells were washed 3 times with DPBS for examination under a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis: Differences between groups were statistically analysed by paired t test and one way analysis of variance (ANOVA) model of SPSS 20, followed by post-hoc analyses to compare each experimental group. P value <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

For high yield cell culture, one needs a medium that supplies the necessary nutrients and growth factors. The chemical composition of the medium is very complex but essentially consists of four groups of components: inorganic salts, carbohydrates, amino acids, and various supplements (i.e. vitamins, fatty acids, lipids, and growth factors). FBS is a significant component of media as it contains many supportive factors for cell maintenance and division (Van Der Valk *et al.* 2018). Considering the effects of FBS supplementation, the effect of different concentration of FBS on total number pSSCs and types of colonies was studied.

In the present study, testicular digestion was performed by using cocktail of enzymes in two steps, obtaining cell viability as high as 90% as previously demonstrated in goat SSCs culture by Pramod and Mitra (2014). Also, co-culture

method for pSSCs culture was used as it is the most commonly used method. There are two culture systems in laboratory for spermatogenesis. First is culturing of testis and second is culturing suspension of isolated cells. Though the first way is interesting but because of complex condition of testis structure and its physiological condition, it is difficult, so the second way is commonly used by researchers (Aponte *et al.* 2005).

As protocol involved double enzymatic digestion with four enzymes (hyaluronidase, collagenase IV, trypsin and DNase I), microscopic observation showed that first enzymatic digestion treatment allowed the separation of seminiferous cords from the interstitial tissue (Fig.1B) while incubation with second enzymatic digestion resulted in release of most of the spermatogonia from the seminiferous epithelium as single cells (Fig.1C).

Furthermore, with differential method of adherence, fibroblasts and other cells started adhering within 4 h after seeding. Also, Differential 2 (D2) was better than differential 1 (D1) on the basis of cluster or larger size colonies in culture. This could be due to reason as this procedure helped in enriching the SSCs by collecting the floating germ cells and transferring them to new plates subsequently. This helped in elimination of somatic cells.

Effect of different concentrations of FBS on types of pSSc colonies: As differential adherence method helped in enriching the SSCs by collecting germ cells, transferring them to new wells subsequently, on day 4, pSSCs were observed in different forms. The colonies were mostly single (Fig. 1D), oval and biconvex. Some of the colonies were observed paired (Fig. 1E), cluster forms (Fig. 1F). On subsequent days of culture, the number of pSSCs in colony increased followed by increase in the size of the colonies finally attaining three-dimensional (3D) rose shape (rosette colony) (Fig. 1G). Cellular connections were also observed between sertoli cells and SSCs colonies (Fig. 1I). Somatic

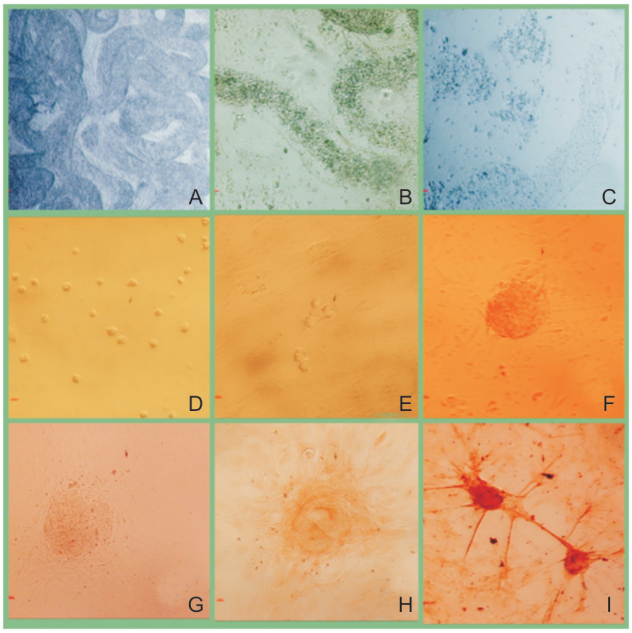

Fig. 1. Isolation of spermatogonial stem cells (SSCs). **A**. Seminiferous tubules before digestion; **B**. Seminiferous tubules after 1st digestion; **C**. Seminiferous tubules after 2nd digestion; **D**. Single SSC colonies; **E**. Paired SSC colonies; **F**. Cluster SSC colony; **G**. Rosette SSC colony; **H**. Rosette colony stained with Alkaline Phosphatase (ALP) and **I**. Cellular connections stained with ALP.

cells proliferated very fast to the formation of monolayer in FBS containing groups. Data is represented in Table 1.

Adherence and proliferation were not observed in serum free medium. Results showed that the rate of cell proliferation and colony formation constantly increased with increase in serum concentration from 5% to 20% FBS group ($P<0.01$). However, further increase in serum concentration resulted in the decline in colony number. This could be due to the reason that growth inhibiting activities

Table 1. Effect of different concentration of FBS on types of putative spermatogonial stem cells

FBS (%)	Method	Colonies			
		Single colonies	Paired colonies	Cluster colonies	Rosette colonies
5% FBS	D1	$24.00^{d}\pm1.51$	$13.20^{bcB}\pm1.15$	$11.40^{bB}\pm1.07$	$0.40^{aB}\pm0.24$
	D2	$15.80^{c}\pm1.39$	$25.60^{dA}\pm1.02$	$25.20^{dA}\pm1.59$	$1.80^{aA}\pm0.37$
10% FBS	D1	$46.00^{eB}\pm1.41$	$30.40^{c}\pm1.43$	$19.80^{bA}\pm1.28$	$7.0^{aA}\pm0.70$
	D2	$34.60^{dA}\pm1.07$	$43.40^{e}\pm1.53$	$55.00^{fB}\pm1.00$	$18.20^{bB}\pm1.28$
15% FBS	D1	$67.60^{e}\pm1.96$	$67.60^{e}\pm1.96$	$46.80^{cA}\pm1.52$	$15.00^{aA}\pm1.00$
	D2	53.80d±1.35	53.80d±1.35	73.40fB±1.32	37.00bB±1.41
20% FBS	D1	$88.60^{d}\pm2.06$	$74.40^{cA}\pm1.28$	$78.40^{cA}\pm1.69$	$41.80^{aA}\pm1.15$
	D2	$74.00^{c}\pm1.41$	$85.80^{dB}\pm0.86$	$96.20^{eB}\pm1.06$	$58.60^{bB}\pm1.93$
25% FBS	D1	$54.00^{fB}\pm1.30$	$40.00^{dA}\pm1.81$	$36.00^{cA}\pm1.41$	$10.00^{a}\pm0.70$
	D2	$47.00^{eA}\pm0.70$	$51.60^{fB}\pm1.12$	$55.00^{fB}\pm0.89$	$14.60^{b}\pm1.32$
30% FBS	D1	$34.40^{eB}\pm1.28$	$26.40^{d}\pm2.97$	$12.40^{bA}\pm0.92$	$0.80^{a}\pm0.37$
	D2	$13.60^{bA}\pm1.32$	$18.40^{c}\pm1.32$	$21.60^{cB}\pm0.81$	$3.40^{a}\pm0.50$
35% FBS	D1	$15.40^{dB}\pm0.50$	$11.60^{c}\pm1.02$	$5.80^{b}\pm0.73$	$0.00^{a}\pm0.00$
	D2	$7.40^{bA}\pm0.67$	$10.00^{c}\pm0.70$	$11.60^{c}\pm0.74$	$1.00^{a}\pm0.54$

$^{a, b, c, d, e, f, g}$Different lowercase superscripts within rows indicate significant differences ($P<0.05$); $^{A, B}$Different uppercase superscripts within rows indicate significant differences ($P<0.05$).

Table 2. Effect of different concentration of FBS on total number of putative spermatogonial stem cell colonies at different passage

FBS (%)	Passage number								
	P1	P2	P3	P4	P5	P6	P7	P8	P9
5% FBS	$44.00^{b}\pm3.72$	$90.66^{b}\pm5.333$	$204.16^{b}\pm11.89$	$270.33^{c}\pm12.29$	$334.33^{c}\pm15.34$	$400.66^{c}\pm9.81$	$129.33^{b}\pm4.8$	$60.83^{c}\pm2.28$	$22.33^{b}\pm2.04$
10% FBS	$91.83^{d}\pm1.42$	$153.00^{d}\pm1.673$	$256.00^{c}\pm5.72$	$289.33^{d}\pm5.00$	$369.66^{d}\pm3.48$	$430.66^{d}\pm5.64$	$178.16^{c}\pm3.48$	$73.16^{d}\pm2.49$	$32.83^{b}\pm1.64$
15% FBS	$171.83^{f}\pm4.75$	$204.66^{f}\pm1.76$	$279.83^{d}\pm4.86$	$312.00^{e}\pm3.30$	$394.83^{e}\pm4.86$	$462.50^{e}\pm7.64$	$227.50^{e}\pm4.48$	$106.50^{e}\pm3.91$	$68.33^{c}\pm3.58$
20% FBS	$204.50^{g}\pm3.17$	$319.33^{g}\pm6.08$	$618.83^{e}\pm5.49$	$739.00^{f}\pm9.48$	$819.83^{f}\pm7.08$	$934.16^{f}\pm9.08$	$511.83^{f}\pm5.47$	$422.66^{f}\pm8.34$	$112.33^{d}\pm7.43$
25% FBS	$141.66^{e}\pm2.9$	$171.83^{e}\pm2.45$	$270.16^{cd}\pm4.96$	$302.33^{de}\pm4.30$	$381.00^{de}\pm3.73$	$448.00^{de}\pm5.7$	$191.16^{d}\pm3.41$	$82.50^{d}\pm2.48$	$58.00^{c}\pm4.38$
30% FBS	$73.00^{c}\pm1.52$	$139.83^{c}\pm2.92$	$198.00^{b}\pm8.91$	$160.66^{b}\pm3.38$	$132.50^{b}\pm1.89$	$91.33^{b}\pm3.41$	41.6667 ± 2.48	$20.16^{b}\pm1.99$	$9.50^{a}\pm1.70$
35% FBS	$10.50^{a}\pm1.17$	$47.66^{a}\pm2.95$	$123.66^{a}\pm7.29$	$64.00^{a}\pm4.36$	$22.16^{a}\pm1.19$	$11.83^{a}\pm1.24$	$6.16^{a}\pm1.07$	$2.83^{a}\pm0.60$	$2.16^{a}\pm0.47$

$^{a,b,c, d, e, f}$Different lowercase superscripts within column indicate significant differences (P<0.05).

might have started due to very high concentration (25–35%). Also, significantly higher (P<0.01) number of colonies was observed in 20% FBS group as compared to all other groups. Accordingly, high concentration of FBS (20%) increased the proliferation of cultured cells relative to low (5%) and standard (10%) concentrations.

We also observed four different types of colonies (single, paired, aligned, cluster and rosette colonies) within two weeks of culture as reported by Heideri *et al.* (2012) and Pramod and Mitra (2014).

Strong cellular connections were also observed between germs cells and somatic cells. These connections provide a suitable niche for colony formation as micro environment *in vitro*. SSCs require a niche by Sertoli cells *in vivo*, probably this microenvironment can be reproduced *in vitro* (Jabarpour and Tajik 2018). Sertoli cells were predominantly polygonal or epithelial-like, but as cultures became confluent, bipolar or fibroblast-like cells appeared.

Effect of different concentration of FBS on total number of pSSCs with respect to passage number: After subsequent passages, most of the seeded cells were adherent on the first day itself. Though the colonies exhibited different morphology (viz. single, paired, cluster and rosette), the single colonies were found to be more initially. Colony formation started from fourth day of culture and maximum number of colonies in all passages was observed in 20% FBS group (Table 2).

In different passages, colonies exhibited different morphology (viz. single, paired, cluster and rosette), however, single colonies were found to be more initially. Culture in 20% FBS gave consistently significantly higher (P<0.05) number of colonies in every passage (from P1 to P9) but after P9, cells in every group showed adipocyte like differentiation. In this study, total number of colonies in every passage increased with increase in serum concentration from 5% to 20% FBS group (P<0.05). However, further increase in serum concentration resulted in the decline in colony number in every passage. Direct cell to cell contact could induce spontaneous differentiation if they are allowed to become confluent above 80%. Hence, in the present study, sub-culturing was done after every 4th day.

Characterisation of pSSCs colonies: Alkaline phosphatase staining showed dark red colour in colonies whereas sertoli monolayer was lightly coloured (Fig. 1H). The response of the colonies to alkaline phosphatase staining was observed under an inverted microscope. Immunocytochemistry result indicated that these cells expressed positive results for OCT 4 and PGP9.5 germ cell marker (Fig. 2).

Strong alkaline phosphatase activity was observed in pSSCs indicating a high expression of alkaline phosphatase whereas the feeder cells were lightly coloured. This is in agreement with the findings of Van Der Wee *et al.* (2001) and Stefkova *et al.* (2015) in mouse. SSCs were also positive for the transcription factor Oct4 and PGP9.5 which was confirmed by intranuclear green fluorescence. Oct4 is

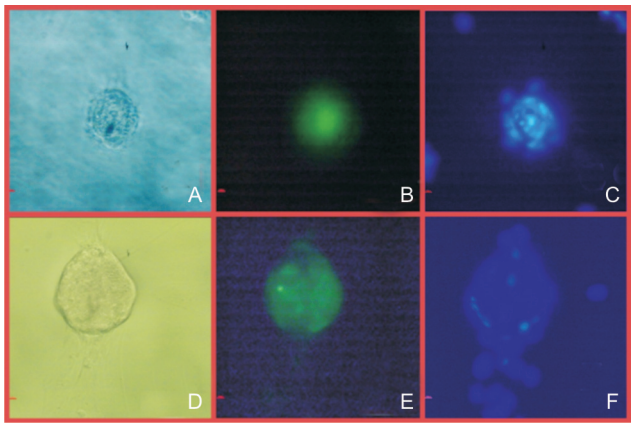

Fig. 2. Characterization of putative Spermatogonial stem cell colonies (SSCs). **A**, **D**. SSC colony in bright field; **B**. Immuno flourescence with intracellular marker OCT 4; **C**. Immuno flourescence with marker PGP9.5 and **C**, **F**. Flourescence with DAPI.

required to govern the pluripotency of cells (Reed and Jhonson 2008) so it indicated that the SSC colonies were in undifferentiated stage and had pluripotent characteristic while PGP9.5 is a protein found in both cytoplasm and nucleus of gonocytes.

In our culture system, SSCs survived under relatively simple culture condition without growth factors, hormones and other unusual component as our primary supplement was fetal bovine serum (FBS). It might have provided all the important biological molecules such as albumin, antichymotrpsin, apolipoproteins, biotin, and growth supporting factors to culture, which are required for optimal growth of cells. Serum also changes the physiochemical properties of the cell culture media, including viscosity, osmolality, buffering capacity, and diffusion rates.

In conclusion, this study revealed that proliferation of SSCs colonies can be modulated by the amount of FBS present in the culture medium. Our data also suggest that the utilization of FBS at a concentration of 10%, which is currently regarded as a 'gold standard' practice in cell culture protocols, is not optimal for cell proliferation. Therefore, we propose that FBS could be readily employed at high concentrations (20%) for enhanced cell proliferation.

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