Indian Journal of Experimental Biology
Vol. 63, May 2025, pp. 389-400
DOI: 10.56042/ijeb.v63i05.15249

Growth factors and culture media dependent *in vitro* expansion and characteristics of enriched spermatogonial stem cells derived from adult caprine testis

Manisha Pathak[1], Shiva Pratap Singh[1]*, Juhi Pathak[1], Anjana Goel[2], Yogesh Kumar Soni[1] & Manoj Kumar Singh[3]

[1]Animal Physiology and Reproduction Division, [3]Goat Genetics and Breeding Division, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura 282 005, Uttar Pradesh, India

[2]Department of Biotechnology, GLA University, Mathura 281 406, Uttar Pradesh, India

Received 22 November 2024; revised 11 February 2025

In vitro expansion of spermatogonial stem cells (SSCs) has gained significant attention, as it offers a promising alternative for preserving and utilizing these cells beyond their natural regenerative capacity. Present study aimed to investigate the effect of supplementation of growth factors in culture media and its comparison with commercially available media for *in vitro* expansion and maintenance of culture characteristics of adult caprine SSCs (cSSCs). In Trial-1, cSSCs were isolated from adult goats and an enriched population of cSSC was randomly divided into 5 groups i.e., group-1 (control; no growth factor), group-2 (GDNF; 40 ng/mL), group-3 (FGF2; 10 ng/mL), group-4 (EGF; 5 ng/mL) and group-5 (GDNF+FGF2+EGF). Following cultivation of cSSCs, morphological assessment, colony counting, and expression of alkaline phosphatase (ALP) and PGP9.5 were conducted and results were compared among the groups. Further, in Trial-2, the performance of optimized cSSCs culture media (in-house media) was compared with 5 commercial media viz, α-MEM, MesenPRO RS™, StemPRO®-34, Stemline®, and Ham's F-12 Nutrient Mix for improved growth and culture characteristics of cSSCs. The cluster-forming activity (CFA) assay, ALP staining, morphological evaluation of cSSCs colonies, and expression analyses of marker genes were performed. In Trial-1, the total number of colonies, size of colonies, and ALP expression were significantly (P<0.05) higher in group-5 compared with other groups. In Trial 2, the in-house media produced significantly (P<0.05) higher number and larger cSSC colonies among all the media tested. Similar results were observed in CFA and ALP staining. The results of expression analyses demonstrate upregulation of pluripotency (PGP9.5 and PLZF) and adhesion (E-cadherin) marker genes, and downregulation of apoptosis marker gene (BCL-6) in the cells when grown in in-house media. Overall, our results demonstrate that in-house media, with a combination of growth factors, provides a more favorable niche for proliferation, colony formation, and maintenance of functional characteristics of adult cSSCs in the *in vitro* culture systems. These results can be utilized for future studies and application that require optimum expansion of cSSCs or other stem cells.

Keywords: Spermatogonial stem cells, Testis, Growth factors, Culture media, Proliferation, Adult goat

Spermatogonial stem cells (SSCs) are a vital component of the testicular environment, responsible for the continuous production of sperm throughout the life of a male[1]. Recent advances in SSC culture, gene editing, and transplantation techniques have opened up new possibilities for using these cells to address various infertility issues. Furthermore, in veterinary medicine, the ability to manipulate SSCs offers exciting prospects for preserving endangered species and improving breeding programs. As research continues to refine the methodologies for SSC isolation and culture, the potential for their clinical application in medicine is becoming increasingly feasible. The integration of SSC-based therapies could revolutionize infertility treatments and contribute to broader regenerative medicine strategies[2]. Given the sparse population of SSCs in the testicular cellular pool and their crucial role in spermatogenesis, understanding the *in vitro* culture characteristics of these enriched cell populations can be instrumental in developing methods for their maintenance and expansion under controlled conditions[3].

Growth factors are integral to the regulation of SSCs, influencing their self-renewal, proliferation, and differentiation, which are critical for sustained spermatogenesis. Recent studies have highlighted the importance of glial cell line-derived neurotrophic factor (GDNF), which remains a central player in promoting SSC self-renewal[4]. Fibroblast growth factor 2 (FGF2) also continues to be a key factor,

*Correspondence:
Phone: +91 90680 08062 (Mob.)
E-mail: shiva.singh@icar.gov.in

synergizing with GDNF to support SSC proliferation and expansion, primarily by modulating the expression of proliferation-associated genes[5]. Additionally, epidermal growth factor (EGF) has been shown to enhance SSC proliferation by activating the ERK pathway, which is essential for SSC maintenance in culture. While GDNF, FGF2, and EGF are key players, the optimal combination of these factors and the specific culture conditions that best mimic the SSC niche are still under investigation.

Existing literature does not adequately address how different culture media affect adult caprine SSCs (cSSCs), particularly in terms of biological processes such as cell proliferation, colony formation, and SSC purity. So, this study was designed to optimize the culture medium and the added effects of growth factors on the proliferation and maintenance of functional properties of cSSCs. To compare various commercially available media with in-house media, firstly, the effects of various growth factors (GDNF, FGF2, and EGF) on growth characteristics of cSSCs was examined (Trial-1) and then this SSC culture media formulation (in-house media) was compared with various commercially available media (Trial-2). Results were compared in terms of cluster-forming activity (CFA) assay, alkaline phosphatase (ALP) staining, type of colonies, and quantitative real-time expression analysis of marker genes.

Materials and Methods

Sample collection

Testes from adult Barbari bucks (n=6) were collected immediately after slaughter from a local abattoir. They were transported to the laboratory within 30 min in normal saline containing antibiotics [penicillin (400 IU/mL) and streptomycin (500 µg/mL)]. Upon arrival, the testes were washed 3 to 4 times with tissue washing medium [Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Cat#14040-117) containing antibiotic-antimycotic solution (10 µL/mL; Sigma-Aldrich, Cat#A5955) and gentamycin (10 µg/mL; Sigma-Aldrich, Cat#G1264)].

Isolation of caprine SSCs (cSSCs)

Using a sharp surgical blade, the tunica albuginea was removed. 4 to 5 g of the exposed testicular parenchyma containing seminiferous tubules were separated from the testicle and were minced in a glass petri dish containing tissue washing medium. The minced tissue was washed in cell-washing medium [DMEM/F-12 (Sigma-Aldrich, Cat#D8437) containing 10% Fetal bovine serum (FBS; Gibco, Cat#10082-147), 1% non-essential amino acid solution (NEAA; Sigma-Aldrich, Cat#M7145), 10 µL/mL antibiotic-antimycotic solution and 10 µg/mL gentamycin] and then subjected to enzymatic digestion and further processing.

Enzymatic digestion

The minced testicular tissue fragments were digested via 2-step enzymatic digestion process. In the process of first enzymatic digestion, the testicular tissue fragments were mixed in DMEM with a blend of digestive enzymes including hyaluronidase (1 mg/mL; Sigma-Aldrich, Cat#H2126), collagenase IV (1 mg/mL; Sigma-Aldrich, Cat#C5138), trypsin (1 mg/mL; Sigma-Aldrich, Cat#T7409), and DNase type I (5 µg/mL; Sigma-Aldrich, Cat#DN25)]. This mixture was incubated at room temperature with continuous shaking in a shaker incubator for 45 min. Following this, the partially digested seminiferous tubules were centrifuged at 100 × *g* for 5 min at 25°C. The supernatant was discarded and a second digestion was performed for 30 min using the same cocktail of enzymes in DMEM. After the second digestion, the tubules were centrifuged again at 100 × *g* for 5 min at 25°C, and the supernatant (cell suspension) was collected for testicular cell harvesting.

Harvesting of cells

For harvesting of cSSCs, the supernatant which contained cSSCs, Leydig cells, myeloid cells, Sertoli cells, and other contaminants from the seminiferous tubules, was filtered sequentially through 80 µm followed by 60 µm mesh filters.

Enrichment of cSSCs

Preliminary enrichment of cSSCs by a differential plating method

A differential plating method was employed to preliminarily enrich cSSCs, as detailed by Singh *et al.*[6]. In this procedure, 24-well cell culture plates (Sigma-Aldrich, Cat#L2766) were first coated with 500 µL *Datura stramonium* agglutinin (5 µg/mL in PBS) and incubated at 37°C with 5% CO_2 for 1 h. After coating, the plates were blocked with DPBS containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Cat#A8860) followed by washing with DMEM supplemented with 0.5% BSA before seeding with the mixed testicular cell population (2.0×10^5 cells/cm²) obtained through two-step enzymatic digestion process. The cells were then incubated overnight in a CO_2 incubator at 37°C and 5% CO_2 to allow for the collection of non-adherent cells. The

following day, the cell population, which primarily included cSSCs, was collected by centrifugation (100 × *g* for 5 min at 37°C) and was pooled for further enrichment using Percoll density gradient centrifugation (PDGC) and magnetic-activated cell sorting (MACS) techniques.

Final enrichment of cells by PDGC and MACS

The additional enrichment of the purified population of cSSCs was done by discontinuous PDGC and MACS as the methods described earlier[7]. Briefly, an iso-osmotic Percoll (Sigma-Aldrich; Cat#P1644) suspension was prepared by adding DMEM with 0.6% BSA and 45 mg/mL DNase type I. Then, the layers of discontinuous density gradient were prepared by the addition of DMEM containing BSA (0.7%) and DNase type I (50 mg/mL) slowly (through the wall) onto the iso-osmotic Percoll suspension. The differential density gradients were prepared by carefully layering 1.0 mL of each Percoll into a 15 mL centrifuge tube. The cells obtained after differential plating were suspended in 500 µL DMEM and were added gradually on top of the density gradient for centrifugation (250 × *g*) for 30 min at 25°C. The 28, 30, and 32% Percoll suspension layers that contain most of the enriched population of cSSCs were collected and pooled for final enrichment of the cSSCs via MACS method. For the final enrichment of cSSCs via MACS, MiniMACS unit (Miltenyi Biotec, Cat#130-042-102), MS column (Miltenyi Biotec, Cat#130-041-201) and Thy1 polyclonal primary antibody (Invitrogen; Cat#BS-0778R) were used according to the method presented earlier by Singh *et al.*[7].

Cell quantification and viability assessment

Prior to Trial-1, the enriched cSSCs were quantified and their viability was assessed using the trypan blue exclusion method. The enriched cell population was first centrifuged at 500 × *g* for 5 min. The pellet was resuspended in a base culture medium and briefly vortexed to achieve a single-cell suspension. To evaluate cell viability, an equal volume of 0.4% trypan blue solution (Gibco, Cat#15250-061) was added to the cell suspension. After incubating the cell suspension for 5 min at Room Temperature (RT, 25°C), 10 µL of the mixture was analyzed for viability (live/dead cells) using the Countess™ automated cell counter following the procedure outlined by Singh *et al.*[8].

Trial-1: Effect of different growth factors

Trial-1 was conducted to evaluate the effect of growth factors on the number and type of SSC colonies. The enriched cSSC population was randomly divided (~1 × 10^6 cells in each group) and grouped as: Group 1 (control): Base culture medium (DMEM/F12 with 15% FBS, 1% NEAA, 10 µL/mL antibiotic-antimycotic solution, and 10 µg/mL gentamycin) without growth factor; Group 2: Base culture medium added with 40 ng/mL GDNF (Sigma-Aldrich, Cat#G1401); Group 3: Base culture medium added with 10 ng/mL FGF2 (Sigma-Aldrich, Cat#F3133); Group 4: Base culture medium added with 5 ng/mL EGF (Sigma-Aldrich, Cat#E4127); Group 5: Base culture medium added with 40 ng/mL GDNF + 10 ng/mL FGF2 + 5 ng/mL EGF.

Colony quantification

To quantify the number of cSSC colonies, approximately 1 × 10^6 cells in each group were cultured under specific conditions for 7 days to allow for colony formation. Post-culture, the colonies, typically spherical or irregularly shaped, were examined and counted at lower magnification (10×) manually under an inverted microscope (Zeiss Axiovert A1, Germany) with a digital camera (Zeiss AxioCam Icm 1) in ten randomly selected fields.

Alkaline phosphatase (ALP) staining

To compare the ALP activity of cSSCs in treatment groups with different growth factors, ALP staining was done via commercial kit (Sigma Aldrich; Cat#86C) and following the manufacturer's instructions. After three time washing of monolayer of cultured cSSCs with DPBS (with Ca^{++} and Mg^{++}), the cell colonies were fixed with the incubation of cultured cell monolayer with citrate (3.12 mL)-acetone (8.12 mL)-formaldehyde (1 mL) solution for 60 seconds., followed by thrice washings by deionized water. Then, the alkaline dye was added to the well and incubated for 15 min at RT. Further, the culture plates were washed thoroughly with deionized water before adding a neutral red stain for 2 min and finally washed with deionized water. The stained colonies were examined under a bright field microscope.

Immunocytochemical (ICC) evaluation

Double immunofluorescence (dIF) labeling was conducted with minor modifications following the protocol described by Singh *et al.*[8]. ~5×10^4 number of enriched cSSCs were seeded in 96-well culture plates in different groups. Once the cells became ~50% confluent, they were permeabilized with Triton X-100 (0.5%, 100 µL/well) and fixed with a fixative solution

(Citrate-Acetone-Formaldehyde solution; 100 µL/well) for 30 min at RT. Then, the blocking solution (2% BSA in PBS, 150 µL/well) was added for 1 h at RT to block unoccupied spaces followed by three 5-min washes with DPBS. Then, the cells were incubated with the primary antibody i.e., mouse monoclonal PGP9.5 (1:100 dilution in blocking buffer), for 1 h at RT. Subsequently, cells were washed three times with DPBS and then treated with a FITC-labeled secondary antibody (anti-mouse IgG) for 30 min at RT, followed by three more washes. Finally, cells were counterstained with DAPI (1 µg/mL) for 1 min and, washed three times with DPBS before being examined under a fluorescence microscope (Zeiss Axiovert A1, Germany).

Trial-2: Comparative evaluation of in-house and commercial cell culture media

The second trial was performed to evaluate and compare the suitability of the in-house media (DMEM/F12, 15% FBS, 1% NEAA, 10 µL/mL antibiotic-antimycotic solution, 10 µg/mL gentamycin supplemented with 40 ng/mL GDNF, 10 ng/mL FGF2, and 5 ng/mL EGF) and five commercially available culture media for improved *in vitro* growth and culture characteristics of cSSCs. For this, the in-house media and commercial cell culture media viz, α-MEM (Gibco, Cat#12561-049), MesenPRO RS™ basal media (Gibco, Cat#12747-010), StemPRO®-34 SFM (Gibco, Cat#10640-019), Stemline® mesenchymal stem cell expansion medium (Merck, Cat#S1569) and Nutrient mixture F-12 Ham (Merck, Cat#N6658) were used.

Evaluation of type of colonies

For evaluation of the type of colonies in different culture media, an equal number of enriched cSSCs (~1 × 10^6 cells) were seeded in a 6-well cell culture plate (SPL Life Sciences, Cat#30006). The growth of colonies was monitored on every alternate day till day-15. The number of single and paired colonies were counted on day-4, whereas a number of cluster and rosette colonies were assessed on day-10 and day-15, respectively.

Cluster forming activity (CFA) assay

The CFA assay was performed in 24-well cell culture plates (SPL Life Sciences, Cat#30024) using crystal violet staining to evaluate the ability of cultured cSSCs to form cell clusters or colonies when cultivated with different growth factors. For this, approximately 1 × 10^6 enriched cSSCs in each group were cultured for 7 days. After incubation for 7 days, the cell colonies were washed with PBS and fixed by treating the cells by 4% paraformaldehyde for 15 min at room temperature. Then, the colonies were washed twice with PBS and stained by incubating fixed cells with 1% crystal violet solution (Sigma-Aldrich, Cat#V5265) for 30 min at room temperature. The stained cell colonies were then washed carefully with distilled water before examined under an upright microscope (Motic; BA410E) equipped with a digital camera (Moticam 4000X). For quantitative analysis, the crystal violet stain was solubilized using a 1% SDS solution for 10 min at room temperature, and the absorbance was measured at 590 nm using a spectrophotometer (Sun Rise, Tecan).

ALP and ICC evaluation

Following the culture of cSSCs in in-house and commercial culture media, ALP staining and characterization of cSSCs for expression of pluripotency marker (PGP9.5) was performed as presented in Trial-1.

Marker genes expression analyses

Total RNA was extracted from the enriched cells cultivated in different culture media for two weeks via an RNAiso Plus and according to the manufacturer's instructions (DSS Takara, Cat#9108). After extraction, the quality and quantity of RNA were measured via nanospectrophotometer (Nanodrop Lite Plus, ThermoFisher Scientific). This RNA was then used for cDNA synthesis with a kit (DSS Takara, Cat#6110A) and a thermocycler (Biorad, C1000™).

The quantitative qRT-PCR was conducted using TB Green® Premix Ex Taq™ (DSS Takara, Cat#RR42LR) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific) to evaluate the differential expression levels of specific marker genes related to pluripotency (UCHL-1 and PLZF), adhesion (E-cadherin), and apoptosis (BL-6) across different treatment groups. Each 20 µL qRT-PCR reaction was performed in duplicate, with 50 ng of cDNA template, 0.5 pmol of each primer, and 0.5 µL of 1× master mix, with an annealing temperature set at 58°C. Controls included No-reverse transcription controls and No-template controls, with GAPDH serving as an endogenous control. The expression level of marker genes was assessed using the Comparative Ct (ΔΔCt) method.

Statistical analysis

Statistical differences among the groups were analyzed using paired *t*-tests and one-way ANOVA

with SPSS 20. Post-hoc tests were performed to compare each trial group. A *P* value of less than 0.05 was considered statistically significant.

Results

Trial 1: Effect of different growth factors

Colony quantification

The effect of growth factors on cSSCs was contrasted by counting total number of colonies on the seventh day. The results indicate the favorable effect of growth factor supplementation on colony formation. The effect of growth factors is more notable when they were used in combination as the growth factors all together encouraged SSC proliferation in culture. This is indicated by a significantly higher ($P< 0.05$) number of total colonies in group 5 than other treatment groups (Fig. 1 & Fig. 2).

ALP and ICC staining

The ALP staining was executed to detect the pluripotent characteristics of cSSC colonies in media with different growth factors (Fig. 2). The outcomes demonstrated that growth factors encouraged cSSC proliferation and cSSC colonies multiplied more than those in the control group (Fig. 2). Representative images of the immunofluorescence staining demonstrating the expression of PGP9.5 in the cSSC

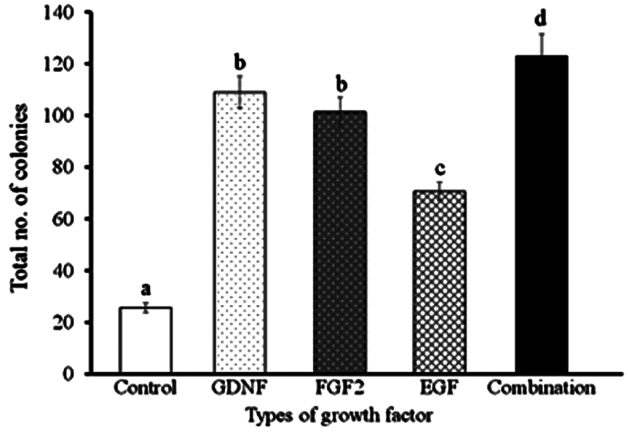

Fig. 1 — Representative images of the effect of growth factors on colony formation and alkaline phosphatase (ALP) staining of cSSC. The ALP staining of cSSC colonies was performed when cells were grown for 10 days in either base media (DMEM/F12) without growth factor (Control) or base media with growth factors [base media + glial cell line derived neurotrophic factor (GDNF), base media + fibroblast growth factor 2 (FGF2), base media + epidermal growth factor (EGF), and base media + combined growth factors (GDNF+FGF+EGF)]. The results indicate a relatively greater number of colonies and higher expression of ALP with addition of growth factors to the base culture medium compared with the control. Arrows indicate cSSC colonies representing ALP staining.

colonies cultivated in the media supplemented with different growth factors are shown in Fig. 3. Relatively higher expression of PGP9.5 was observed in the colonies when cells were cultivated in the culture media with combination of the growth factors.

Trial 2: Comparative evaluation of in-house and commercial cell culture media

Evaluation of type of colonies

To determine the most efficient medium for expansion of cSSC in culture, we proceeded to evaluate the efficiency of our in-house media and distinct commercially available cell culture media. In all of the cell culture conditions, there was no discernible cytotoxic effect during the cultivation process. The enriched population of cSSCs was grown in different culture media (in-house media, MEM, MesenPRO, StemPRO, Stemline, and Nutrient F12 media) and displayed four types of SSC colonies (single, pair, cluster and rosette). The representative images of the cSSCs when the enriched population of cSSCs were expanded for 15 days during primary culture in different culture media is presented in Fig. 4. The number of all 4 types of colonies in in-house media was significantly higher ($P< 0.05$) than other groups. Furthermore, paired, cluster, and rosette colonies were not observed in Nutrient F12 media (Fig. 5).

CFA assay, ALP, and ICC staining

The results of CFA assay indicate that the in-house media significantly ($P< 0.05$) improved the cluster formation of cultured cSSCs compared with the other media tested (Fig. 6A**)**. While comparing the absorbance among the groups, a significantly ($P< 0.05$) higher values were detected with in-house media (1.49 ± 0.02) when compared with StemPRO®-34 SFM (1.31 ± 0.03), α-MEM (1.27 ± 0.02), Stemline®Mesenchymal Stem Cell Expansion Medium (1.21 ± 0.02), and MesenPRO (1.17 ± 0.01). The results also demonstrate that significantly ($P< 0.05$) lower values of absorbance were observed with Nutrient Mixture F-12 Ham (0.46 ± 0.03) among all groups (Fig. 6B). Similarly, the outcome of ALP and ICC staining suggests higher expression of ALP and PGP9.5 in the cSSCs when grown in the in-house media than in other cell culture media (Fig. 7 & Fig. 8).

Differential expression analyses of marker genes

A comparison of differential expression of transcripts for pluripotency (PGP9.5 and PLZF),

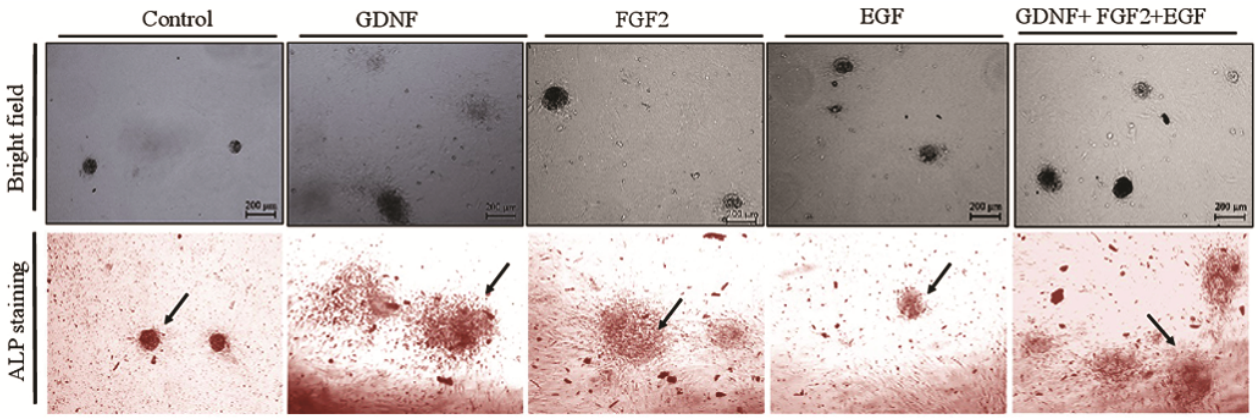

Fig. 2 — Graphical representation of total number of cSSC colonies when enriched cSSCs were cultivated for 7 days in the base medium (DMEM/F12) supplemented with or without growth factors. Group 1 (Control; base medium without growth factor); Group 2: Base medium supplemented with 40 ng/mL glial cell line derived neurotrophic factor (GDNF); Group 3: Base medium supplemented with 10 ng/mL fibroblast growth factor 2 (FGF2); Group 4: Base medium supplemented with 5 ng/mL epidermal growth factor (EGF); Group 5 (Combination): Base medium supplemented with 40 ng/mL GDNF + 10 ng/mL FGF2 + 5 ng/mL EGF. The different letters over the bars (a, b, c, and d) represent significant ($P< 0.05$) differences among the groups.

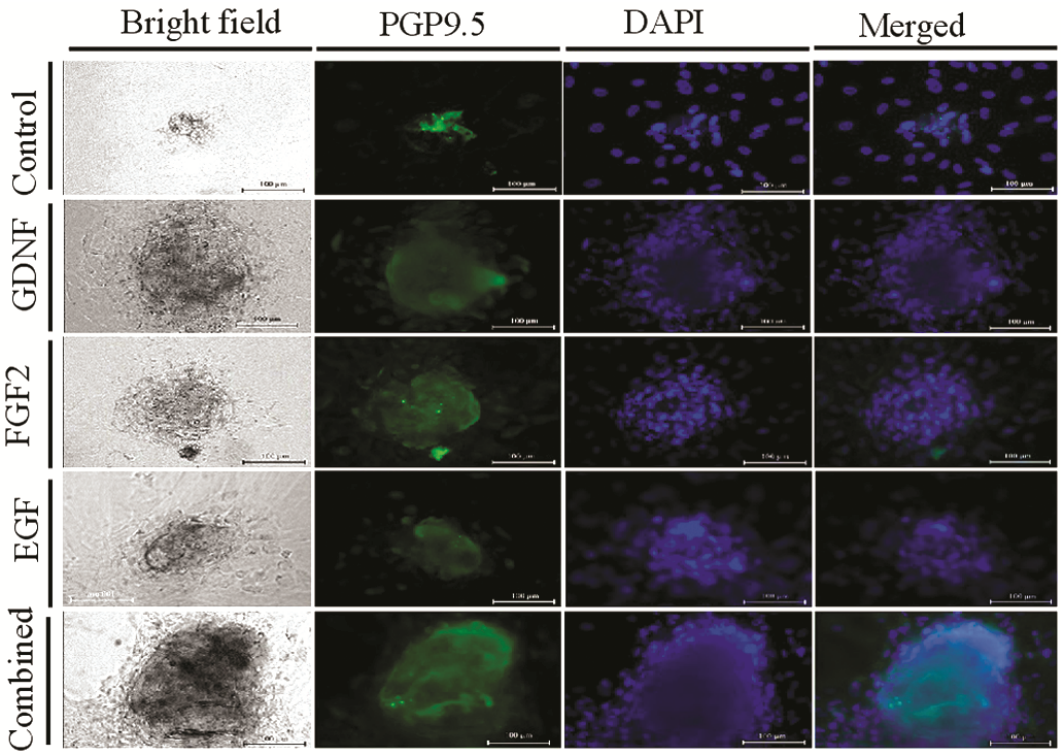

Fig. 3 — The representative images of immunocytochemical characterization of cSSCs colonies via immunostaining with pluripotency specific marker (PGP9.5, protein gene product 9.5) in different treatment groups. Relative fluorescence positivity was observed in the cSSC colonies grown in media with different growth factors. Control = Base culture media (DMEM/F12) without growth factor, GDNF = Base culture media with GDNF (40 ng/mL), FGF2 = Base culture media with FGF2 (10 ng/mL), EGF = Base culture media with EGF (5 ng/mL), and Combination = Base culture media with GDNF, FGF2, and EGF. Scale bar = 100 µm.

adhesion (E-cadherin), and apoptosis (BCL-6) specific marker genes in the cultured cSSCs after 10 days of cultivation in different culture media is presented in Fig. 9. The results indicate upregulation of pluripotency (PGP9.5 and PLZF) and adhesion (E-cadherin) marker genes, and downregulation of apoptosis marker gene (BCL-6) in the cells when grown in in-house media. These outcomes

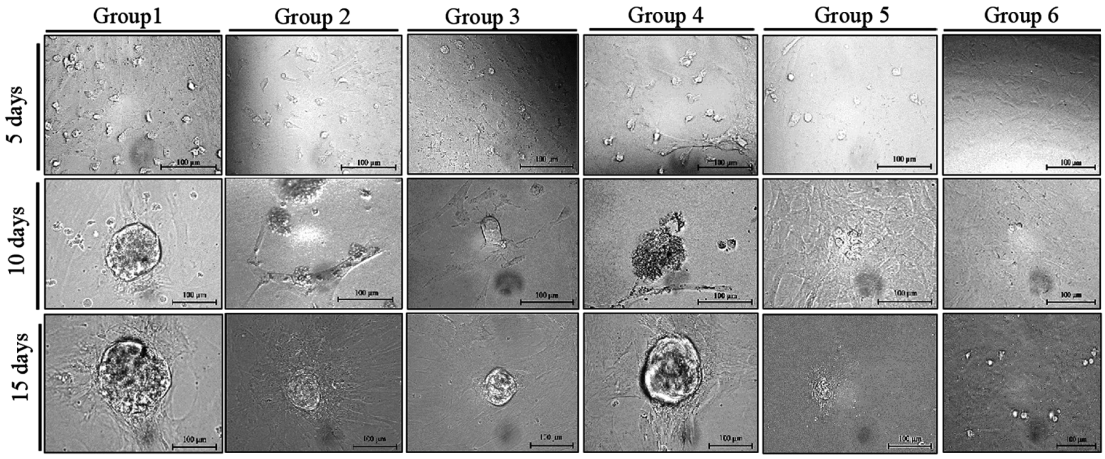

Fig. 4 — Representative images of the cSSCs when the enriched population of cSSCs were expanded for 15 days during primary culture in the respective culture media. Group 1 = In-house, Group 2 = MEM, Group 3 = MesenPRO, Group 4 = StemPRO, Group 5 = Stemline and Group 6 = Nutrient F12. Largest colonies were observed in Group 1 and Group 4. Scale bar = 100 µm.

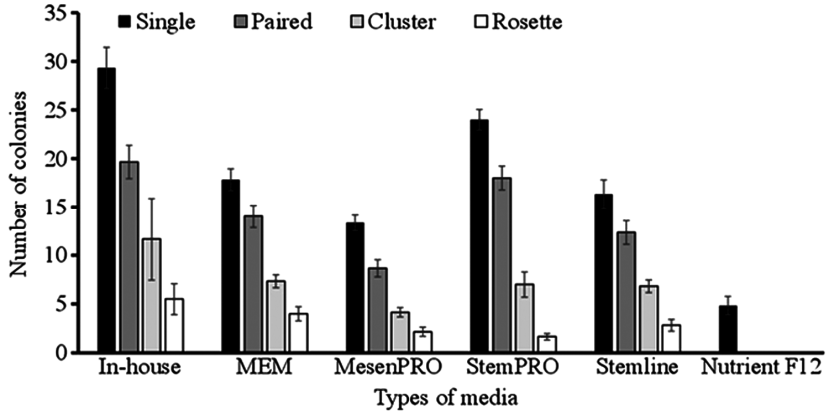

Fig. 5 — Representative images of the cSSCs when the enriched population of cSSCs were expanded for 15 days during primary culture in the respective media. Group 1 = In-house, Group 2 = MEM, Group 3 = MesenPRO, Group 4 = StemPRO, Group 5 = Stemline and Group 6 = Nutrient F12. Largest colonies were observed in Group 1 and Group 4.

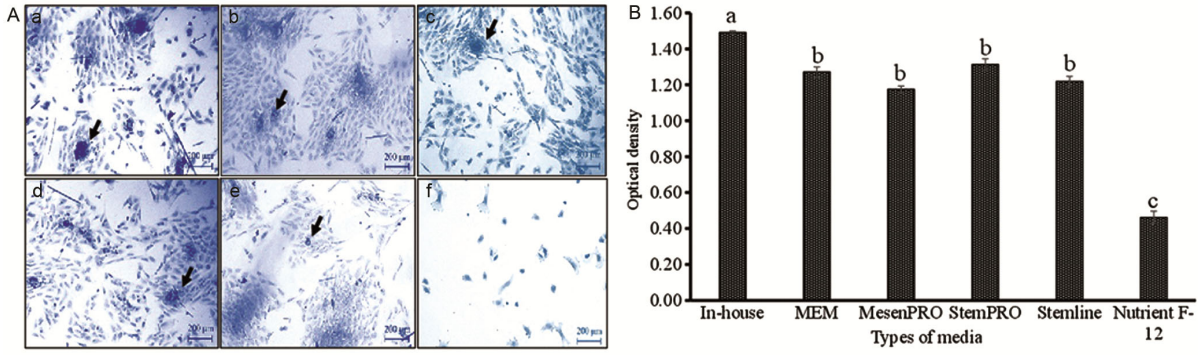

Fig. 6 — Comparative evaluation of the effect of culture media on the cluster-forming activity (CFA) of cSScs. (A) The enriched population of cSSCs were grown in different culture media (In-house, MEM, MesenPRO, StemPRO, Stemline, and Nutrient F12 media) for 7 days. After incubation for 7 days, the cell clusters were washed with 1× PBS and fixed with 4% paraformaldehyde for 15 minutes at RT. Following fixation, the clusters were stained with 1% crystal violet solution for 30 minutes at RT. After washing, the clusters were examined under an inverted microscope. (B) Comparison of optical densities showing the effect of different culture media on CFA efficiency of cSSCs. Optical density was measured after solubilization of stain (crystal violet) with 1% SDS solution.

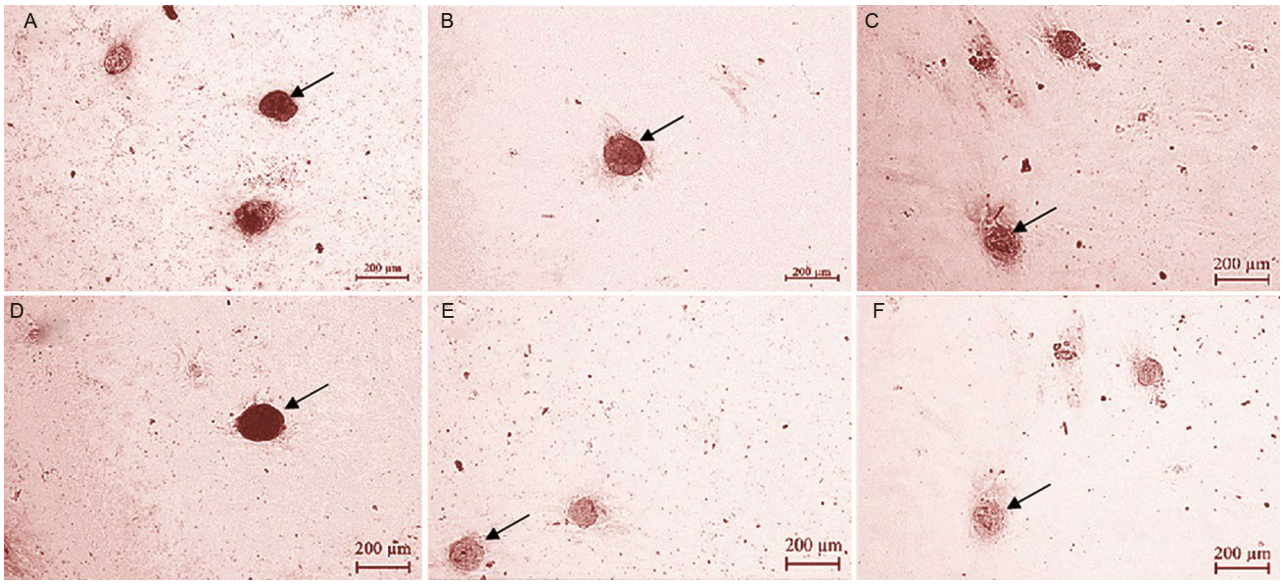

Fig. 7 — The representative images of crystal violet staining of cSSCs culture in respective groups after 7 days in culture. The optical density after solubilization of crystal violet in respective groups. Bars with different letters are significantly different ($P< 0.05$). Scale bar, 200 µm. Arrowheads indicate individual colonies. A = In-house medium, B = MEM medium, C = MesenPRO medium, D = StemPRO medium, E = Stemline medium, and F = Nutrient F12 medium.

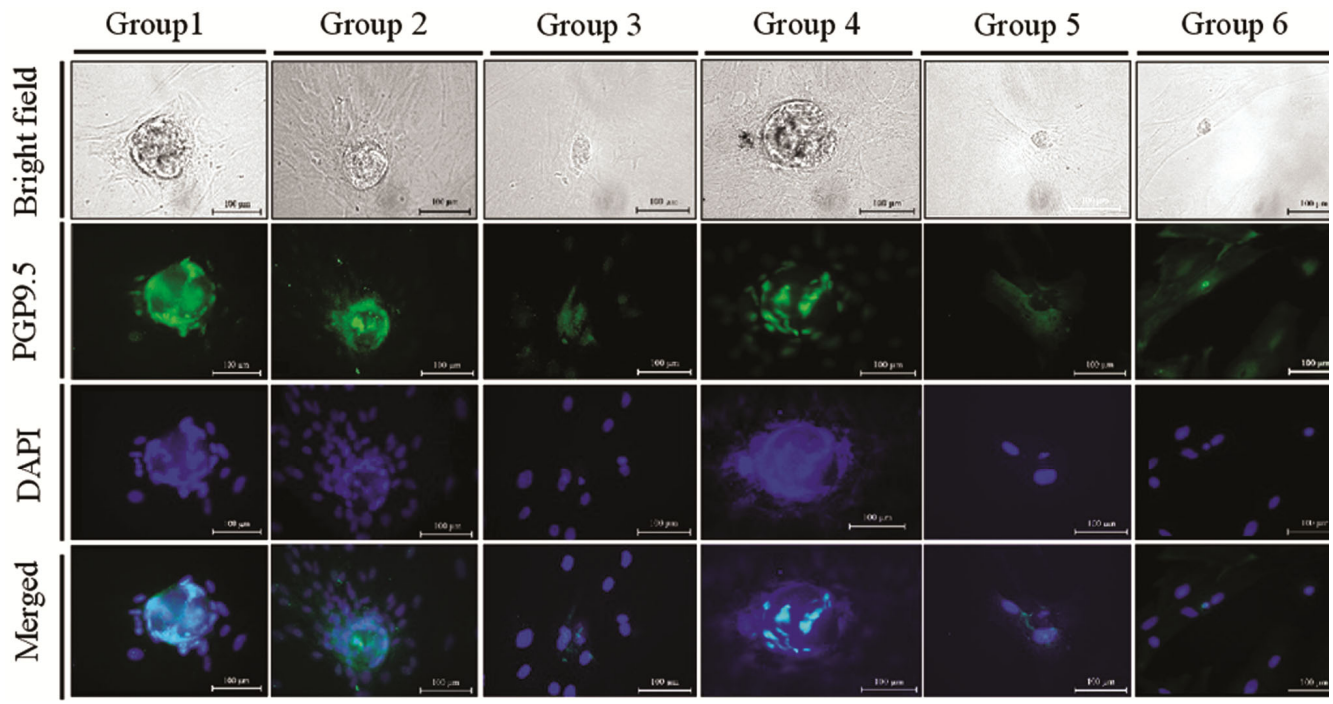

Fig. 8 — The representative images for characterization of cSSCs colonies via immunostaining with SSC specific marker (PGP9.5, protein gene product 9.5) in different treatment groups. Relative fluorescence positivity was observed in the cSSC colonies grown in different growth factor media. Group 1 = In-house medium, Group 2 = MEM medium, Group 3 = MesenPRO medium, Group 4 = StemPRO medium, Group 5 = Stemline medium and Group 6 = Nutrient F12 medium. Scale bar = 100 µm.

demonstrated a more favourable microenvironment when cSSCs were grown in in-house media compared with the commercial cell culture media tested.

Discussion

Spermatogonial stem cells (SSCs) represent a promising avenue for regenerative medicine,

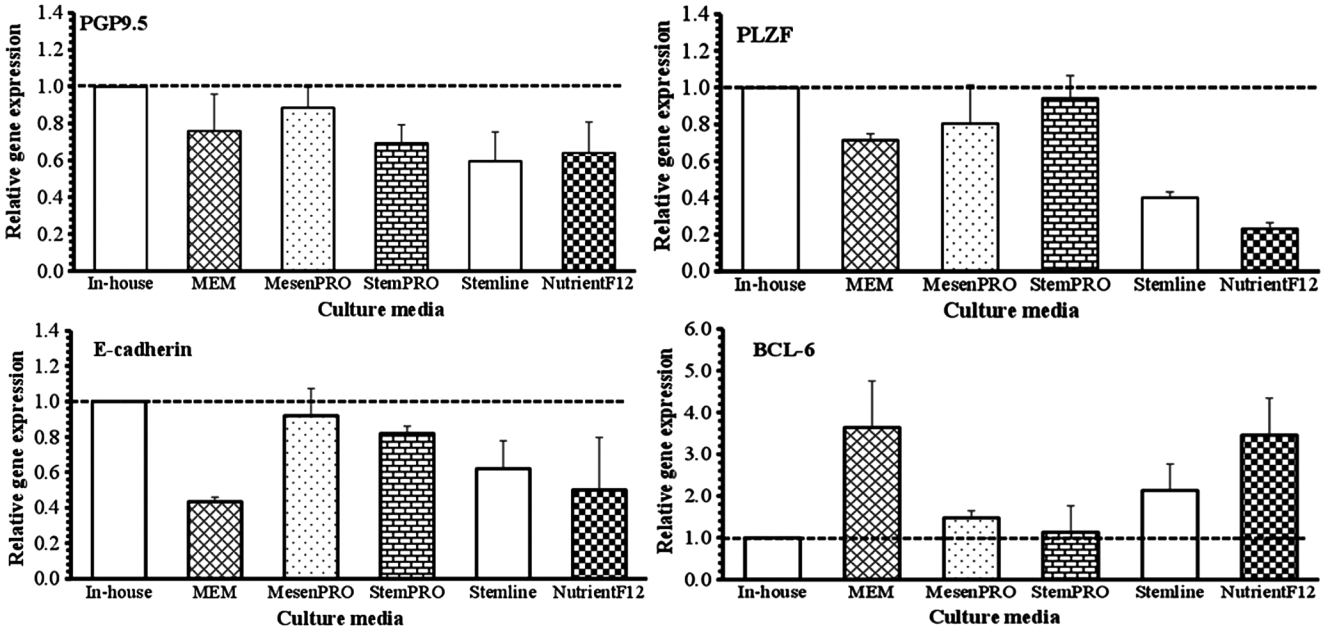

Fig. 9 — Comparison of transcription levels of pluripotency, adhesion, and apoptosis specific marker genes in the cultured cSSCs after 10 days of cultivation in different culture media. The transcription levels of genes were estimated using real-time qPCR considering DMEM supplemented with growth factors as a control. The culture of cSSCs in MEM and other media resulted in the down-regulation of specific markers of pluripotency (PGP9.5 and PLZF), and adhesion (E-cadherin). Whereas, the expression of the apoptosis related gene (BCL-6) was up-regulated in almost all the treatment groups. Error bars represent standard error (SE), n = 2. When compared with the control the significant difference in other groups is indicated by * $P< 0.05$, ** $P< 0.01$. PCR = polymerase chain reaction; PGP9.5, protein gene product 9.5 (alternate designation: UCHL-1, ubiquitin carboxyl-terminal esterase L-1), PLZF = promyelocytic leukemia zinc finger; and BCL-6 = B cell lymphoma 6.

particularly in the treatment of male infertility and the regeneration of damaged testicular tissue. SSCs possess the unique ability to both self-renew and differentiate into spermatozoa, making them invaluable for restoring spermatogenesis in individuals with compromised fertility. The selection of appropriate culture media is critical for maintaining the biological traits of SSCs and enabling their differentiation into various cell types. Culture media provide the essential nutrients, growth factors, and microenvironment conditions (niche) that influence the fate of SSCs. Changes in media composition can significantly affect the self-renewal capacity, viability, and differentiation potential of SSCs. The specific combinations of growth factors in the culture media have been shown to promote SSC proliferation while maintaining their undifferentiated state. Understanding how media formulations affect SSCs properties is crucial for optimizing their use in regenerative medicine and infertility treatments.

The present study demonstrates the effect of growth factor supplementation and different cell culture media on variables related to the *in vitro* characteristics of enriched and cultured cSSCs viz. colony formation, types of colonies, CFU, ALP, ICC and quantitative real-time expression analyses of marker genes. When exploring the growth factor supplementation on colony formation, according to the results of the study, colony formation was significantly higher ($P< 0.05$) with growth factor supplementation. Specifically, the combination of growth factors (GDNF+ FGF2+EGF) significantly increased ($P< 0.05$) the number of cSSC colonies, indicating a robust effect on cSSC proliferation and maintenance. Several factors, including laboratory conditions, base media, serum source, handling, cell counting techniques, and the inclusion of growth factors and media supplements can influence the expansion of cultured cells and their colony formation.

GDNF is crucial for maintaining the undifferentiated state of SSCs, preventing premature differentiation while promoting long-term self-renewal. Furthermore, FGF2 plays a critical role in supporting SSC self-renewal by enhancing cell proliferation and survival. Conversely, EGF synergistically enhances the effects of FGF2 and GDNF, further promoting cell division and colony formation. This combination of factors created an optimal culture condition that improved the efficiency of SSC expansion and contributed to their stemness and

differentiation potential[9]. Moreover, our results are in agreement with other studies, depicting the combined presence of these growth factors in the culture medium significantly increases SSC proliferation rates and maintains their stemness, enabling the efficient expansion of SSC lines *in vitro*[10].

Furthermore, the culture of cSSCs often results in the formation of distinct colony morphologies, including single cells, paired cells, clusters, and rosettes. These colony types reflect different stages of SSC self-renewal and differentiation. Single and paired colonies are typically indicative of undifferentiated SSCs, maintaining their stemness and proliferative capacity. As cSSCs begin to differentiate or aggregate, they form clusters, which can include a mix of SSCs and progenitor cells. Rosette formations are more organized structures, often associated with higher differentiation and signaling interactions within the cells. These morphological distinctions are critical for understanding SSC biology and optimizing culture conditions for SSC maintenance and expansion. This study highlighted the influence of different culture media on the type of colonies based on their morphological characteristics. Results of all media displayed all four types of SSC colonies except in Nutrient F12 media which displayed only single colonies. Indicating, that the composition of the culture medium, including the presence of growth factors, significantly influences SSC proliferation, self-renewal, and differentiation. Furthermore, a greater number of single and paired colonies in in-house media indicates that it supports the maintenance of cSSCs in an undifferentiated state, leading to the formation of single and paired colonies. The fine-tuning of culture medium components is essential for controlling the balance between SSC self-renewal and differentiation, making it a critical factor in SSC culture systems.

Furthermore, the role of the culture medium is pivotal in dictating the efficiency and nature of cluster formation. The culture medium provides the necessary nutrients and growth factors that support the proliferation and self-renewal of cSSCs. The specific composition of the medium influences the ability of cSSCs to aggregate into clusters, which are often indicative of a balance between self-renewal and early differentiation. Key components like GDNF, βFGF and EGF are essential for maintaining SSCs in an undifferentiated state, thus promoting robust cluster formation. As per our results, a significantly higher ($P< 0.05$) number of cluster formations was observed in in-house culture medium when compared with other culture media. Thus, the culture medium is not just a passive environment but an active modulator of cSSC behavior, significantly impacting the outcomes of cluster-forming assays.

As the culture medium also plays a pivotal role in maintaining their stemness and influencing differentiation, these effects the expression of ALP activity (a marker of pluripotency) and SSC-specific markers. The choice of culture medium, including its components like growth factors, directly impacts the expression of these markers. Results of this study showed high expression of ALP and SSC-specific markers in all groups studied. Recent studies have demonstrated that optimized media formulations, which often include growth factors such as GDNF, FGF, and EGF, enhance the expression of SSC markers and sustain ALP activity, thereby promoting the proliferation and survival of SSCs while preventing spontaneous differentiation[11].

These results are in agreement with prior investigations that have demonstrated the synergistic effects of growth factors such as GDNF, FGF2, and EGF in promoting the proliferation and sustaining the undifferentiated state of SSCs in vitro. A study by Binsila *et al.*[10] demonstrated that, supplementation with GDNF and EGF significantly improved proliferation and stemness of SSCs up to 7 days in culture. GDNF at 40 ng/mL maintained proliferation and stemness for 36 days, outperforming other growth factors tested. This study also highlighted that EGF at 15 and 20 ng/mL exhibited comparable proliferation and stemness in vitro as detected by the putative stem cell marker PLZF, GFRα1, and ITGA6. These findings suggest that GDNF and EGF play crucial roles in SSC proliferation and maintenance in vitro.

Furthermore, Kanatsu-Shinohara *et al.*[12] reported that SSCs cultured in culture medium supplemented with GDNF, soluble GFRα1, and β-FGF exhibited continuous proliferation for over six months without loss of function, and the cultured SSCs retained their capacity to reconstitute spermatogenesis upon transplantation into recipient testes, indicating the preservation of their functional properties. It has been observed that while GDNF and FGF2 individually support SSC proliferation, their combined application results in a significantly greater expansion of SSC populations, indicating a synergistic interaction between these growth factors[13].

Moreover, studies have also demonstrated that FGF2 regulates the self-renewal of spermatogonial stem cells (SSCs) via specific intracellular signalling pathways. FGF2 has been shown to induce the expression of ETV5, along with GDNF, in Sertoli cells through activation of the MAPK and PI3K signalling cascades. The transcription factor ETV5, predominantly expressed in Sertoli cells, is essential for SSC maintenance, and its absence leads to impaired self-renewal and progressive germ cell depletion[14]. This work contributes to a better understanding of how to manipulate culture environments to control SSC fate, which has implications for both basic research and clinical applications in reproductive biology and regenerative medicine.

Conclusion

The in-house medium [cell culture medium (DMEM/F12) with a combination of growth factors (GDNF+FGF2+EGF)] is the most appropriate media for the cultivation of cSSCs compared with other tested media supplemented with either a single growth factor or without any growth factor. While comparing in-house medium with commercially available cell culture media, the highest number and the largest size of cSSC colonies, highest expression of pluripotency markers (PGP9.5 and PLZF), coupled with significantly higher expression of the adhesion marker gene (E-cadherin) and downregulation of apoptosis marker gene (BCL-6) suggest more favorable niche while cultivation of cSSCs using in-house media. The outcome of the present study is important for replicating physiological conditions in experimental models for future research involving cSSCs or any other stem cells, which can lead to future developments in tissue engineering and regenerative medicine.

Ethical statement

All the experiments were conducted following the guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)," Government of India. The Institutional Animal Ethics Committee (IAEC) duly approved the present investigation (IAEC/CIRG/Sr Nr. 6/2019).

Funding statement

The study was supported by a grant from the National Livestock Mission (NLM) (Grant No. R-99011(11)9/2022-DADF-Dept (E-23298)), Government of India, New Delhi.

Acknowledgment

The authors are thankful for the support extended by the director of the Institute for providing the necessary facilities to carry out this study.

Conflict of interest

The authors of this study affirm that there were no financial or commercial affiliations that may be viewed as having a possible conflict of interest.

References

1 Diao L, Turek PJ, John CM, Fang F & Reijo Pera RA. Roles of spermatogonial stem cells in spermatogenesis and fertility restoration. *Front Endocrinol*, 13 (2022) 895528.

2 Kanatsu-Shinohara M & Shinohara T. Spermatogonial stem cell self-renewal and development. *Annu Rev Cell Dev Biol,* 29 (2013) 163–187.

3 Singh SP, Kharche SD, Pathak M, Soni YK, Pawaiya RV, Quadri SA, Singh MK & Chauhan MS. Establishment of effective and safe recipient preparation for germ-cell transplantation with intra-testicular busulfan treatment in pre-pubertal Barbari goats. *Theriogenology*, 189 (2022) 270–279.

4 Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A & Shinohara T. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. *Stem Cell Reports*, 4(3) (2015) 489–502.

5 Morimoto H, Ogonuki N, Kanatsu-Shinohara M, Matoba S, Ogura A & Shinohara T. Spermatogonial stem cell transplantation into nonablated mouse recipient testes. *Stem Cell Reports*, 16(7) (2021) 1832–1844.

6 Singh SP, Kharche SD, Soni YK, Pathak M, Ranjan R, Majhi SK, Pawaiya RS, Singh MK & Chauhan MS. Successful in vivo transplantation of cultured and enriched testicular germ cells of pre-pubertal bucks to busulfan-treated homologous recipients. *Cells Tissues Organs*, 212(3) (2023) 232–244.

7 Singh SP, Kharche SD, Pathak M, Soni YK, Gururaj K, Sharma AK & Singh MK, Chauhan MS. Temperature response of enriched pre-pubertal caprine male germline stem cells *in vitro*. *Cell Stress and Chaperones*, 26(6) (2021) 989–1000.

8 Singh SP, Kharche SD, Pathak M, Ranjan R, Soni YK, Singh MK, Pourouchottamane R & Chauhan MS. Low oxygen tension potentiates proliferation and stemness but not multilineage differentiation of caprine male germline stem cells. *Molecular Biology Reports*, 48(6) (2021) 5063–5074.

9 Liu W, Du L, Li J, He Y & Tang M. Microenvironment of spermatogonial stem cells: a key factor in the regulation of spermatogenesis. Stem Cell Res Ther, 15 (2024) 294.

10 Binsila BK, Selvaraju S, Ghosh SK, Ramya L, Arangasamy A, Ranjithkumaran R & Bhatta R. EGF, GDNF, and IGF-1 influence the proliferation and stemness of ovine spermatogonial stem cells *in vitro*. *J Assist Reprod Genet*, 37(10) (2020) 2615.

11 Tan L, Liu Q, He Y, Zhang J, Hou J, Ren Y, Ma W, Wang Q & Shao C. Establishment and characterization of a spermatogonial stem cell line from tiger puffer fish (Takifugu rubripes). *Animals*, 13(18) (2022) 2959.

12 Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S & Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod,* 69 (2003) 612–616.

13 Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A & Shinohara T. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. *Stem Cell Rep,* 4 (2015) 489–502.

14 Liu W, Du L, Li J, Zhang X, Wang F, Zhao H, Li Z, Chen J, Yang L, Huang Y, Luo L, Wu J, Wei L, Lin T, Zhang S, Shi Y, Yang X, Liu Y, Li Q, Zhao L, Zhang J & Wang Y. Microenvironment of spermatogonial stem cells: a key factor in the regulation of spermatogenesis. *Stem Cell Res Ther,* 15 (2024) 294.