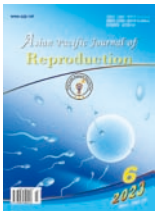

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Cryopreserved ovine spermatogonial stem cells maintain stemness and colony forming ability *in vitro*

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ABSTRACT

Objective: To assess the effect of cryopreservation on stemness and proliferation potential of sheep spermatogonial stem cells (SSCs) *in vitro*.

Methods: Sheep testicular cells were isolated and putative SSCs were enriched by the laminin-based differential plating method. Putative SSCs were co-cultured with the Sertoli cell feeder prepared by the Datura Stramonium Agglutinin (DSA-lectin)-based method. The cultured putative SSCs were cryopreserved in Dulbecco's Modified Eagle Medium-10% fetal bovine serum mixture (DMEM-10% FBS) media containing 10% dimethyl sulfoxide (DMSO) alone or 10% DMSO plus 200 mM trehalose. Cryopreserved putative SSCs were evaluated for their proliferation potential using *in vitro* culture and stemness by immunocytochemistry. Finally, the transfection ability of cryopreserved putative SSCs was analyzed.

Results: We isolated 91% viable testicular cells from sheep testes. The majority of the laminin enriched cells expressed the SSC related marker, ITGA6. Co-culture of sheep putative SSCs with Sertoli cell feeder resulted in the generation of stable colonies, and the expression of SSC marker was maintained after several passages. A significantly higher number of viable putative SSCs was recovered from SSCs cryopreserved in media containing 10% DMSO and 200 mM trehalose compared to 10% DMSO alone (P<0.01). Cryopreserved putative SSCs formed colonies and showed SSC marker expression similar to the non-cryopreserved putative SSCs. The appearance of green fluorescent colonies over the Sertoli cell feeder indicated that cryopreserved sheep SSCs were successfully transfected.

Conclusions: Cryopreserved putative SSCs can retain their stemness, colony forming ability, and transfection efficiency *in vitro*. Our research may help in the effective preservation of germplasm and the generation of transgenic ovine species.

KEYWORDS: Spermatogonial stem cell; Sheep; Stemness; Cryopreservation; Transfection

1. Introduction

Spermatogenesis is a complicated mechanism that is initiated by spermatogonial stem cells (SSCs), which contribute to the transfer of genetic material to future generations in males. Intricate signals to enhance spermatogenesis are mediated by Sertoli cells, which provide nutrition for SSCs and developing germ cells. The *in*

Significance

Despite the higher significance of sheep spermatogonial stem cells (SSCs) in livestock transgenesis, there is a dearth of research to optimize SSC cryopreservation and assess the direct effects of cryopreservation on *in vitro* cultured sheep SSCs. The findings of the current study suggest that the developed cryopreservation technique can effectively preserve the stemness, proliferative potential, and transfection efficiency of cryopreserved sheep SSCs.

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vitro self-renewal of SSCs depends critically on the production of basic fibroblast growth factor (bFGF) and glial cell line-derived neurotropic factor (GDNF) by Sertoli cells[1,2]. Enriching and culturing SSCs would be the prerequisite for any application of these cells, given that SSCs only make about 0.02-0.03 percent of all testicular germ cells[3]. A multi-step enzymatic digestion of the testicular tissues using combinations of two or more enzymes is required for the extraction of SSCs[3,4].

SSC is a fantastic tool for genetic modification, producing transgenic animals effectively[5]. SSC transplantation into a recipient testis or intracytoplasmic sperm injection can result in the production of genetically modified progeny[6-8]. SSC transplantation also has the ability to preserve the germplasm of important livestock breeds.

Cryopreservation and long-term culture techniques have also been employed for the preservation of SSCs. Izadyar and colleagues showed that maintaining pure populations of SSCs in a culture system for an extended period of time is not possible[9]. However, it has been discovered that combining the culture technique with cryopreservation is an effective strategy for the long-term preservation of SSCs[10]. Cryopreservation minimizes the genetic change in continuous lines, reduces transformation in finite lines, diminishes contamination, and prevents the consequences of aging. In mice, early studies on SSC transplantation showed that SSCs may restore spermatogenesis in recipient testes after cryopreservation[11]. Later studies in a greater number of animal species have shown that SSCs are capable of withstanding long-term cryopreservation[12-14]. Recently, Onofre and co-workers demonstrated that cryopreservation of testicular tissue is a suitable method to cryopreserve SSCs prior to SSC transplantation in a mouse[15].

Sheep is currently a crucial model animal in agricultural, pharmacological, and biological research due to its ideal size, short gestation period, and low maintenance cost. The development of new technologies including cell culture, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and transgenic technology has been facilitated by the sheep industry's economic significance. Currently, research on sheep has made substantial use of genetic engineering technology. Despite the greater significance of sheep SSCs in sheep transgenesis, there have only been a limited number of attempts to optimize SSC cryopreservation and directly assess the effects of cryopreservation on *in vitro* cultured sheep SSCs. Novel sheep SSC cryopreservation techniques must be created and improved in order to maximize the effectiveness of future applications. Therefore, the aim of the present investigation was to assess the effect of cryopreservation on the stemness and proliferation potential of sheep SSCs *in vitro*. Furthermore, research was conducted to assess the transfection efficiency of cryopreserved SSCs *in vitro*.

2. Materials and methods

2.1. Animals and testicular cell isolation

Prepubertal male nondescript sheep testes (n=6) were collected from the local abattoir and brought in phosphate-buffered saline (PBS) to the lab within 2 h. The testes' extra fat was completely removed with sterilized scissors. The testes were repeatedly rinsed in PBS that contained 50 μg/mL Gentamicin Sulfate (Sigma, USA). The testis was cut into small pieces after the tunica albuginea was removed. The testicular pieces were sequentially digested with 1 mg/mL collagenase Ⅳ, DNase Ⅰ, and trypsin-EDTA as per the previously reported protocol[4]. Finally, after digestion, the testicular cell suspension was filtered *via* cell strainers made of 100- and 40-mm nylon (BD, USA). The viability of cells was evaluated by Trypan blue staining.

2.2. Feed layer preparation and enrichment of SSCs

For Sertoli cell feeder preparation, first the enzymatically isolated testicular cells were incubated on a Datura Stramonium Agglutinin (DSA-lectin; 10 μg/mL)-coated 6-well plate for 2 h at 37℃ in an atmosphere that was humidified with 5% CO_2. An equal amount of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 (Gibco, USA), containing 5% (v/v) fetal bovine serum (FBS), essential amino acids (EAA; Gibco, USA), non-essential amino acids (NEAA; Gibco, USA), and antibiotic-antimycotic solution, was used as culture medium. Sertoli cells that were adherent were grown for 7-9 days after the non-adherent cells were discarded. Once the Sertoli cells had reached 90% confluency, they received a 3 h treatment with mitomycin C (10 μg/mL). Further, the cells were used as a feeder layer for SSCs after going through three to four rounds of DMEM/F12 washing.

SSCs were purified from testicular cells using the differential plating technique. The suspended testicular cells were cultured in DMEM/F12 with 5% FBS on the laminin-coated plates (20 μg/mL; Sigma, USA) for 2 h at 37℃ in a CO_2 incubator with 5% CO_2. Unattached cells were removed, and attached cells were then trypsinized for 5 min at 37℃ using 0.025% (v/v) trypsin-EDTA. The detached cells were washed, and the pellet was suspended in DMEM-FBS media.

2.3. In vitro culture of SSCs

The cells obtained from differential plating (1×10^5 cells per well) were co-cultured with a Sertoli cell feeder layer using DMEM-F12 containing 10% (v/v) FBS, 1× EAA, 1× NEAA, 15 ng/mL recombinant human GDNF (Thermo Fisher Scientific, USA), 10 ng/mL recombinant human bFGF (Sigma, USA) and 1× antibiotic-antimycotic solution. Every alternate day, the medium was changed. The SSC colonies were gently passaged by pipetting every 10 to 12

days without disturbing the feeder cells. Further, we counted and photographed colonies under 10× and 40× magnification of an inverted phase contrast microscope (Leica Microsystems, Germany).

2.4. SSC cryopreservation

After the 4th passage, SSCs (0.5×10^6/mL) were resuspended separately in two separate cryopreservation media. The first medium involved 10% dimethyl sulfoxide (DMSO) (v/v) and 10% (v/v) FBS in DMEM. The second medium contained 10% DMSO (v/v) (Sigma, Germany), 10% (v/v) FBS, and trehalose 200 mM (Sigma, USA) in DMEM. The diluted cell suspensions were then transferred into 1.80 mL cryotube vials after equilibrating in freezing media for 5 min at 4℃. The cryovials were placed at -80℃ for 4 h and further stored in liquid nitrogen until required. After one month, the cryovials were taken out of the liquid nitrogen storage and kept in a 37℃ water bath. The thawed SSC suspension was pelleted in prewarmed DMEM by centrifugation (500×*g*, 5 min). The resuspended cells in DMEM were used for vitality analysis by trypan blue staining.

To determine the culture ability of freeze-thawed SSCs, the cryopreserved SSCs were co-cultured with Sertoli cell feeder. The culture condition was similar to the condition used for non-cryopreserved SSCs.

2.5. Immunocytochemistry

An immunocytochemistry (ICC) investigation was performed on enriched SSCs and the colonies generated from cryopreserved and non-cryopreserved SSCs. The enriched cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS before being smeared onto a glass slide that had been coated with poly-L-lysine. Further, the slides were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS. After blocking, cells were incubated for 2 h with rabbit polyclonal anti-TGA6 (1:50) primary antibody, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit IgG (1:200) secondary antibody. The cells were examined under a fluorescent microscope after being counterstained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). For the ICC analysis of SSC culture, 6-well plates containing cells were fixed with 4% (w/v) PFA. Here, rabbit anti-ITGA6 (1:100) and FITC-conjugated anti-rabbit IgG (1:200) were used as primary and secondary antibodies, respectively. In the ICC analysis of the Sertoli cell feeder, mouse monoclonal anti-vimentin (1:40) was used as the primary antibody, and FITC-conjugated chicken anti-mouse IgG (1:100) was utilized as the secondary antibody.

2.6. Assessment of transfection efficiency of cryopreserved SSCs

The transfection mixture containing Lipofectamine 2000 (Invitrogen, USA) and linearized pEGFP-N1 plasmid (Clontech, USA) was formulated in DMEM as per the manufacturer's protocol. After being mixed with the frozen-thawed SSC suspension (2×10^5 cells), the mixture was incubated for 60 min at 37℃ with 5% CO_2. After the incubation period, the cells underwent two rounds of washing by being suspended in DMEM at 500×*g* for 5 min. Following the final wash, the pellet was suspended in a fresh culture medium and then cultured on the feeder layer.

2.7. Statistical analysis

All experiments in this study were repeated at least three times. GraphPad Prism (version 7; Graphpad Software Inc, USA) software was used for statistical analysis. Differences between groups were statistically analyzed by one-way ANOVA or Student's *t* test. All the values were expressed as mean±standard deviation (mean±SD). A *P*-value<0.05 was defined as statistical significance.

2.8. Ethics statement

Since this study did not involve any live animals or human, ethics committee permission for conducting the experiment was not required. Handling of animal tissue followed international, state, institutional guidelines.

3. Results

3.1. Isolation and laminin–based purification of ovine SSCs

Sheep testicular cells were isolated by sequential enzymatic digestion with three enzymes, *viz*., collagenase Ⅳ, DNase Ⅰ, and trypsin. Trypan blue staining revealed vitality in the vast majority (91%) of the isolated cells. The laminin-based differential plating method was employed to enrich the putative SSCs from testicular cells. Further, the SSC marker, ITGA6, was used to determine the stemness of the enriched cells. Round to polygonal-shaped putative SSCs with conspicuous nucleus expressed ITGA6 on their surface (Figure 1).

3.2. SSC colony formation on Sertoli cell feeder layer

Sertoli cell as a feeder was used for putative SSC culture. Testicular cells were first cultured on lectin-DSA-coated plates to prepare Sertoli cells. The Sertoli cells were grown in culture, and within 8 to 9 days, they confluently formed a layer (Figure 2A). By evaluating the expression of the Sertoli cell marker vimentin, the cultivated Sertoli cells were further described (Figure 2B).

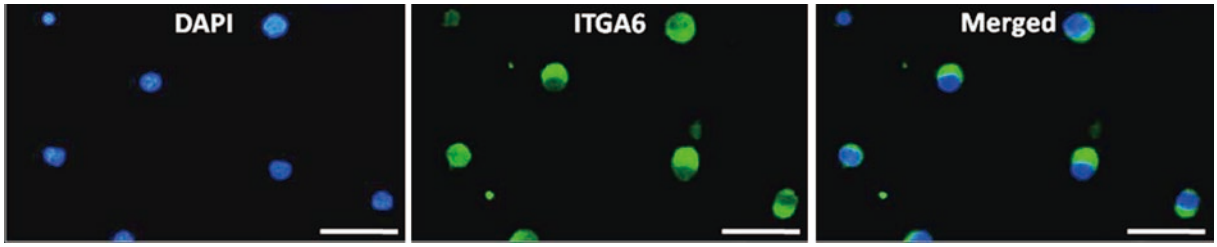

Figure 1. Characterization of enriched sheep spermatogonial stem cells (SSCs) using immunocytochemistry. Most of the enriched cells express the SSC marker, ITGA6. Nuclei (blue) are stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 20 μm.

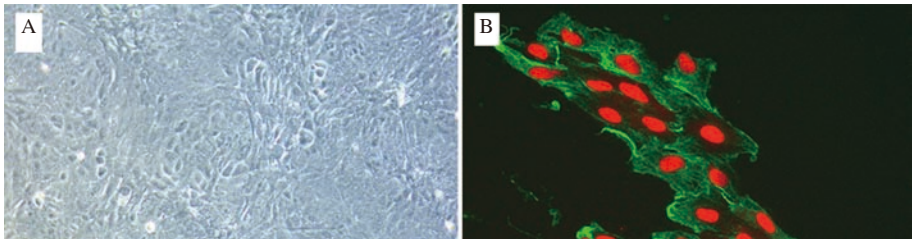

Figure 2. Culture and characterization of ovine Sertoli cells. (A) Primary cultivation of Sertoli cells from sheep testes. Sertoli cells form a confluent layer within 8 to 9 days of culture. Scale bar, 50 μm. (B) Immunostaining of cultured Sertoli cells from sheep testes with an anti-vimentin antibody. Nuclei (red) of cells are stained by PI.

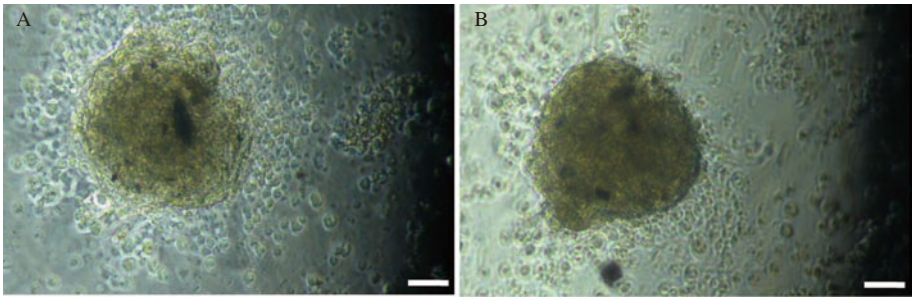

Figure 3. Phenotypic characteristics of SSC colonies generated by co-culturing of sheep SSCs on Sertoli cell feeder. (A) Colony generated from non-cryopreserved sheep SSCs. Sheep SSCs form stable colonies on the Sertoli cell feeder. (B) Colony generated from cryopreserved sheep SSCs. Similar to the non-cryopreserved SSCs, cryopreserved sheep SSCs also form colonies on the feeder. Scale bar, 100 μm.

The enriched putative SSCs were co-cultured with the mitomycin C-treated Sertoli cell feeder. Putative SSCs began to form cell clusters after 48 h, and colonies became apparent after 5 days of co-culture (Figure 3A). Over four passages (P4), the cultivated SSCs maintained their proliferation rate steady and their cell viability high [(88.50±1.44)%]. The results of our ICC analysis revealed that practically all of the SSC colonies expressed the SSC marker, ITGA6 (Figure 4A).

3.3. Recovery rate and proliferation efficiency of cryopreserved SSCs

Sheep SSCs collected after P4 were cryopreserved for a month. After freeze-thawing, the viability of SSCs was assessed employing trypan blue and further analyzed their colony forming efficiency by *in vitro* culture. Significantly higher (P<0.01) numbers of viable SSCs were recovered from SSCs cryopreserved in media containing 10% DMSO and 200 mM trehalose [(75.75±3.594)%] compared to 10% DMSO alone [(66.00±4.163)%] (Figure 5). However, the survival rate of cryopreserved cells in DMSO plus trehalose was significantly lower (P<0.01) than in the non-cryopreserved group [(88.50±2.887)%].

We also looked into the ability of cryopreserved (10% DMSO and 200 mM trehalose group) sheep SSCs to establish colonies. Cryopreserved SSCs formed stable colonies like non-cryopreserved SSCs (Figure 3B). The expression of the SSC-associated marker ITGA6 in cryopreserved SSCs grown on the Sertoli cell feeder was next examined. Most of the cells in SSC colonies expressed ITGA6, as indicated by the Figure 4B.

3.4. Foreign gene transfer to the SSCs

We investigated the transfection efficiency of cryopreserved sheep SSCs. Lipofectamine 2000 was used to carry the foreign gene to the SSCs. After the 5th day of gene transfer, green fluorescent SSC colonies above the Sertoli cell feeder indicated successful gene transfer (Figure 6A). We observed that [(54.00±9.22)]% of SSC colonies expressed *EGFP*. Fluorescent SSCs were absent in the control group (Figure 6B).

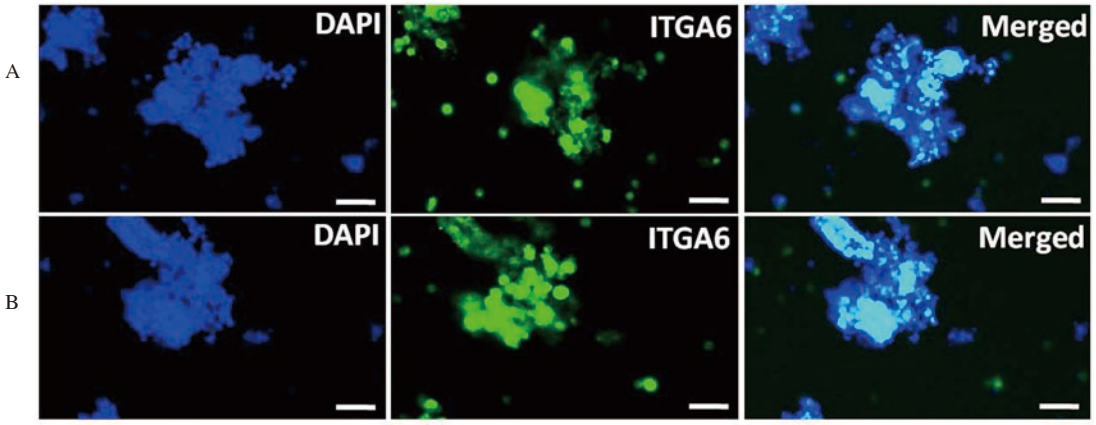

Figure 4. Immunocytochemical analysis of sheep SSC colonies above the Sertoli cell feeder. The SSC marker, ITGA6 expressed in (A) SSC colonies developed from non-cryopreserved sheep SSCs and (B) SSC colonies developed from cryopreserved sheep SSCs. Scale bar, 50 μm.

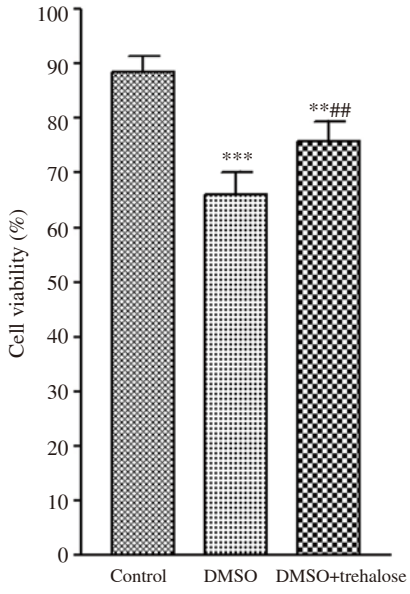

Figure 5. Percentage of viable SSCs recovered after freeze thawing. Differences between groups are assessed by one-way ANOVA. $^{**}P<0.01$, $^{***}P<0.001$: compared to the control group (non-cryopreserved). $^{\#\#}P<0.01$: compared to the 10% DMSO group.

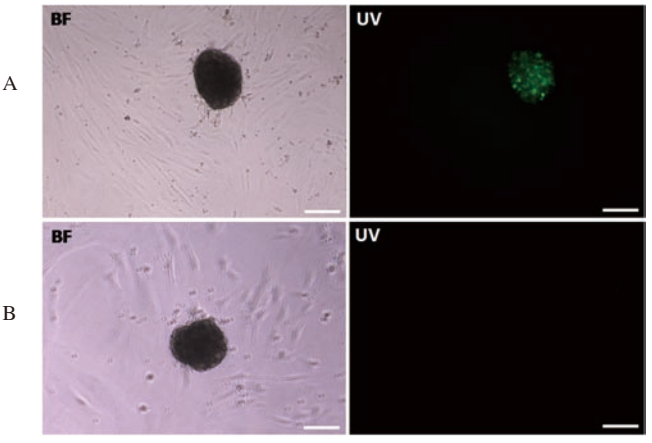

Figure 6. Transfection of sheep SSCs *in vitro*. (A) Green fluorescent colonies indicate *EGFP* gene transfer to the SSC using lipofectamine. Green fluorescent SSC colonies are observed above the Sertoli cell feeder after 5th day of transfection. (B) Green fluorescent SSCs are absent in the control (non transfection) group. Scale bar, 50 μm.

4. Discussion

In the current study, we assessed the effect of cryopreservation on the proliferation potential and stemness of cultured sheep SSCs. First, sheep testicular cells were isolated by sequential enzymatic digestion with three enzymes, *viz*., collagenase Ⅳ, DNase Ⅰ, and trypsin. In the past, with varying degrees of success, attempts were made to isolate the testicular cells in farm animals using either four[16,17], three[4], or two[18] enzymes. In this study, enzymatic isolation resulted in 91% viable testicular cells. Our results were consistent with the results reported in humans[19] and goats[4]. A recent study demonstrated the isolation of 72% viable sheep testicular cells using collagenase and trypsin enzymes[20].

The SSC population has been purified using various extracellular matrix (ECM) types, including laminin, fibronectin, collagen types Ⅰ and Ⅳ, and gelatin[21,22]. Researchers have reported enhanced purifying efficiency of laminin-based methods in mice, rats, bulls and rams[2,23,24]. It has been hypothesized that laminin's great efficacy for SSC selection is related to its receptors on SSCs[25]. The key laminin receptor, ITGA6, is required for the attachment of SSCs to laminin[25]. For the enrichment of sheep SSCs, we employed the same laminin-based differential plating approach as the prior report in sheep[24]. In the ICC examination, almost all of the isolated cells displayed the SSC marker ITGA6. Earlier studies have used ITGA6, PLZF, and GFR1 as markers for the identification of sheep SSCs[20,26].

According to published research, Sertoli cells can be used as a feeder for successful SSC cultivation in goats[4], buffaloes[27] and pigs[28]. The Sertoli cells might be a promising option, as reported in our earlier study, where it was shown that goat SSCs could

survive for one and a half months *in vitro* when co-cultured with goat Sertoli cells[4]. Therefore, as a feeder for the SSC culture in the current investigation, we used sheep Sertoli cells. Sertoli cells release GDNF, which affects the maintenance and proliferation of SSCs[29]. Additionally, SSC' integrin receptors are bound by laminin, which is released by Sertoli cells. Sertoli cells, SSCs, and niche paracrine factors interact specifically, which controls the proliferation and differentiation of SSCs. The Sertoli cells' basal and perinuclear regions contain vimentin, which radiates toward the apical cytoplasm, where it is linked to some specialized membrane connections called "desmosome-like junctions" between Sertoli cells and nearby germ cells[30]. Previously, Qasemi-Panahi and co-workers employed vimentin to characterize sheep Sertoli cells *in vitro*[26].

According to our findings, when Sertoli cells and sheep SSCs were cocultured, numerous SSC colonies appeared. On the fifth day of culture, we noticed SSC colonies on the Sertoli cell feeder. In a previous study, we discovered that goat SSC colonies began to form on the fourth day of culture[4]. A significant increase in the number and diameter of SSC colonies were noticed in accordance with the earlier reports[4,31]. The stemness of the cultured SSCs was indicated by the expression of ITGA6 in SSC colonies.

We evaluated the impact of cryopreservation on the viability, colony-forming abilities, and stemness of sheep SSCs because SSC cryopreservation is important for the generation of transgenic sheep. In our investigation, the survival percentage of cryopreserved sheep SSCs in the 10% DMSO group was (66.00±2.08)% after one month of cryopreservation. Izadyar and co-workers reported successful cryopreservation of bovine spermatogonia in a medium containing glycerol, or DMSO[9]. Comparing the results with propanediol, glycerol, and ethylene glycol (EG), DMSO preserved immature mouse testicular tissue better[32,33]. Viability rates were higher with DMSO and EG, although DMSO was observed to improve graft recovery and reinitiate spermatogenesis following transplantation[32,33]. However, other research suggested that prolonged exposure to high concentrations of DMSO during the vitrification process could have harmful effects and activate the caspase apoptotic pathways; as a result, inhibiting caspase activity would increase the vitality of cells after thawing[34-36].

Trehalose and other sugars can considerably improve the recovery, viability, proliferative ability, and colonization efficiency of mammalian undifferentiated spermatogonia in cryopreservation media[37,38]. Cryopreservation in conditions containing 200 mM trehalose and DMSO has been shown by Lee and co-workers to be a more effective cryoprotectant for murine SSCs than DMSO alone[37]. Kim and co-workers employed slow freezing to cryopreserve putative pre-pubertal bovine SSCs[39]. According to their findings, cryopreservation of bovine SSCs in the presence of 200 mM trehalose is an effective method[39]. Similar to earlier publications in other species, we found that sheep SSCs in cryopreservation media containing both 200 mM trehalose and 10% DMSO had a much greater vitality rate than those in media containing 10% DMSO alone.

Further, we examined the ability of cryopreserved (10% DMSO and 200 mM trehalose group) sheep SSCs to form colonies. We observed that freeze-thawed SSCs developed stable colonies on the Sertoli cell feeder. The number and diameter of SSC colonies were comparable to those generated from SSCs that had not been cryopreserved. Earlier, Koruji and the team demonstrated the *in vitro* colony formation of cryopreserved adult mouse SSCs on the Sertoli cell feeder layer[40]. Hermann and co-workers revealed that DMSO-cryopreserved rhesus macaque SSCs had retained their capacity to engraft[41]. The culture potential, capacity to colonize recipient testes, ability to contribute to viable offspring, and normal spermatogenesis were all retained in PEG cryopreserved mouse SSCs[37].

The use of SSC in the development of genome-edited animals is gaining more and more attention from researchers. Transgenic spermatozoa with donor genotype origins can be produced by intra-testicular transplantation of SSC carrying appropriate transgenes. The generational gap for producing transgenic animals is shortened *via* gene transfection into germline stem cells. Cryopreservation and long-term *in vitro* culture help to save the SSCs for future transfection works. However, there are fewer reports of these areas of research in ovine species. Therefore, in the current study, we used an EGFP reporter to evaluate the transfection of sheep SSCs that had been cryopreserved in 10% DMSO and 200 mM trehalose. Lipofectamine-based transfection resulted in the appearance of fluorescent SSC colonies after the 5th day of culture. Thus, our study suggested that cryopreservation did not affect the colony forming ability of transfected sheep SSCs. The effective transfection of bovine SSC with an EGFP plasmid was reported by Tajik and co-investigators[42]. Recently, a research team revealed that *EGFP* gene transfer into goat SSCs using lipofectamine is more effective than electroporation[43].

While the study's strengths lie in its evaluation of the stemness, proliferation potential, and *in vitro* colonizing ability of cryopreserved sheep SSCs, its comprehensiveness may be limited by the lack of experiments exploring the ability of cryopreserved SSC to colonize recipient testes, contribute to normal spermatogenesis and produce viable offspring.

In conclusion, results of the present investigation suggest that cryopreservation of sheep SSCs with the protocol reported here can maintain the proliferation potential and stemness of SSCs *in vitro*. Further, the transfection efficiency of cryopreserved ovine SSCs *in vitro* is demonstrated. Cryopreservation has no adverse effect on the colony forming ability of transfected sheep SSCs. As there is a significant lack of scientific knowledge with regard to optimal cryopreservation techniques for sheep SSC, the results generated in the study provide a baseline for further research and utilization of these methods in gene delivery, genome editing, and transgenesis in ovine species.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Authors' contributions

R. Kumar Pramod designed the study, conducted experiment, wrote the manuscript and helped in analysis and interpretation of the data. Deepthi Varughese helped in experiment and conducted the data analysis. A. Javed Jameel helped in both interpretation of data and manuscript editing. Bhisma Narayan Panda helped in experiment and manuscript writing. Soma Goswami helped in manuscript writing. Abhijit Mitra was involved in critical reading of the manuscript. All authors read and approved the final manuscript.

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