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Effect of poly-D-lysine on proliferation and senescence of caprine spermatogonial stem cells *in vitro*

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Spermatogonial stem cells (SSCs) have the unique ability of both self-renewing and to produce progeny that undergoes differentiation to spermatozoa. As SSCs exist in very low numbers, therefore efficient *in vitro* expansion of SSCs is important prior to their clinical applications. In this study, we tried to improve the functionality of putative SSCs (pSSCs) during culture using poly-D-lysine (PDL) coating. For this, plates were coated with 0.01% PDL with different coating time interim treatments (5, 30 and 60 min) while control remained uncoated. The adequate amount of pSSCs of the goat was isolated and enriched using two-step enzymatic digestion and differential plating methods. Further, the functionality of pSSCs was evaluated by cell growth analysis, cell proliferation, senescence, and the presence of pluripotency (alkaline phosphatase, OCT-4) and SSC related (PGP-9.5) markers. The number and size of pSSCs colonies in 0.01% PDL coating groups were significantly (P <0.05) higher than in the control group. Similarly, pSSCs on uncoated plates expressed significantly (P <0.05) higher β-galactosidase expression indicating higher senescence of pSSCs on uncoated plates than PDL-coated plates. The results suggest that PDL provides a favourable microenvironment for pSSCs culture as indicated by early attachment, proliferation, and decreased senescence. This strategy may significantly contribute to the efficient culture of pSSCs for further studies of the regulatory mechanism of pSSCs proliferation and differentiation.

Keywords: Aging, Goat, *Capra hircus*

Spermatogonial stem cells (SSCs) as the main undifferentiated cells found in the germline are competent in exchanging genetic information to the next generation and provide a basis for spermatogenesis[1] and male fertility[2]. As putative SSCs (pSSCs) have the ability of self-renewal and differentiation, they are useful for clinical applications[3]. As SSCs hold a great promise for the treatment of infertility problems in men due to premature loss of male germ cells caused during chemotherapy and treatment of other diseases. These SSCs can also be used for propagation and conservation of the genetic profiles of valuable male animals, such as endangered species[4]. The SSCs have therefore become an emerging model for regenerative medicine[5], animal conservation[6], and production of transgenic animals with desired production characterstics[7].

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The SSCs are very rare in number and comprise only 0.02-0.03% of all germ cells in the rodent testis[8,9] and unique marker(s) for SSCs have not been identified yet. Therefore, several approaches to enrich stem cells and *in vitro* culture systems for SSCs in mammals have been attempted[10]. Moreover, neonatal or prepubertal testes are the preferred source for isolation and *in vitro* culture and downstream functional applications of SSCs[6]. The knowledge about the isolation and culture of SSCs would provide a valuable resource to study mechanisms associated with their differentiation and spermatogenesis[11]. A culture system that supports efficient enrichment and proliferation of cells is required to obtain an adequate number of viable cells for clinical experiments. Furthermore, in contrast to embryonic undifferentiated cells, SSCs have a limited life span and stop multiply at some point during *in vitro* culture because of senescence[12]. Senescence in SSCs may also affect its regenerative capabilities[13] indicating the urgent need for the studies to minimize senescence for

effective cell growth and subsequent successful therapeutic application. Therefore, this study was undertaken to develop an optimized culture condition that delays the senescence of pSSCs in the culture system.

Cell adhesion is essential in SSCs cell culture for propagation and development. Poly-D-lysine (PDL) is a small natural homopolymer of the essential amino acid (D-lysine) of extracellular matrix (ECM) proteins. As PDL is known to improve cell adhesion to solid substrates, it can be used for coating purposes and to re-establish the *in vitro* microenvironment. Although the efficiency of PDL in selecting SSCs in caprine has not yet been examined, the PDL-coated plate has been demonstrated to improve the purifying efficacy of gonocyte isolation in bovine[14]. We hypothesized that the use of PDL coating would be advantageous for SSC culture expansion and would protect SSCs properties *in vitro*. In this study, we have compared and analyzed the properties of pSSCs which were cultured onto either uncoated plates or 0.01% PDL-coated plates with different coating time interims (5, 30 and 60 min).

Material and Methods

Isolation and enrichment of putative spermatogonial stem cells (pSSCs)

Goat testes were collected from pre-pubertal bucks (3-6 months of age) immediately after the slaughter from a local abattoir. Testes were taken to the laboratory within 1.0 h in normal saline solution fortified with streptomycin (500 µg/mL) and penicillin (100 IU/mL). All the experimental procedures were carried out following good veterinary practices and approved by the Animal Ethics Committee of the Institute.

After 3-4 times washing with Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Cat#D5773), the tunica albuginea was then detached with a surgical blade and 4-5 g of the testicular parenchyma was 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[15], with some minor modifications. Briefly, After mincing in the sterile Petri dish, testicular parenchyma (~2 g) was suspended in DMEM (5-fold v/v) containing hyaluronidase type II from sheep testes (1.0 mg/mL; Sigma-Aldrich, Cat#H2126), collagenase IV (1.0 mg/mL; Sigma-Aldrich, Cat#C5138), DNase type I (5 µg/mL; Sigma-Aldrich, Cat#DN25), trypsin (1.0 mg/mL; Sigma-Aldrich, Cat#T7409) and antibiotic-antimycotic solution (1% w/v, Sigma-Aldrich, Cat#A5955) before incubated in an orbital shaker incubator for 45 min. Supernatant after washing at 1000 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 the same concentration of digestive enzymes (trypsin, collagenase, hyaluronidase, and DNase) for 30 min, as described above. The supernatant after centrifugation, was filtered through 80 µm (Merck Millipore, Cat#NY8002500) and then through 60 µm (Merck Millipore, Cat#NY6002500) nylon mesh filters to remove Sertoli cells, myeloid cells, and other contaminating cells, and enrich the pSSCs population.

Freshly isolated pSSCs were cultured into 24-well cell culture plates (Cellstar, Greiner) for 24 h in DMEM medium (ThermoFisher Scientific, Gibco, Cat#12660012) with 15% FBS (ThermoFisher Scientific, Gibco, Cat#10082147), gentamycin sulfate (50 µg/mL), antimycotic solution antibiotics (10 µL/mL), non-essential amino acids (1%), and L-Glutamine (1%) until the full attachment of somatic cells to the bottom of the plate in primary culture medium [passage (P)-0] was observed. In differential (D1), after adherence of somatic cells, the floating cells were gently transferred in a fresh well with culture medium and then incubated at 37°C in a humidified atmosphere with 5% CO_2. The culture media was replaced every third day. This strategy of differential adherence was accomplished for enriching the stem cell population. The enriched cell population was further cultured *in vitro* for 10-12 days to obtain 70-80% confluency.

Experimental design

As the present study evaluates the effect of PDL on cell proliferation and senescence, uncoated and PDL (0.01% w/v; Sigma-Aldrich, Cat# P7280) coated 24-well cell culture plates with various time interims were used for culture. Gr. I (Control): 24-well cell-culture plates without PDL coating; Gr. II _IV, PDL coating with 5, 30 and 60 min incubation, respectively

Briefly, after 70-80% confluency, cells were removed from 24-well plates using 0.25% trypsin-EDTA (ThermoFisher Scientific, Gibco, Cat# 25200072) for 4-5 min at 37°C and were seeded into a new coated plate for following passages. The

experiment was conducted from P-1 onwards onto the PDL coated culture plates and the cells were cultured up to P-7. For coating purposes, an aqueous solution of 0.01% PDL was added to the wells. The plates were air-dried for 3 different time interims (5, 30 and 60 min) in a CO_2 incubator at 37°C and stored at 4°C under sterile conditions until used. The cultures were maintained until the 80-90% confluency and the number and types of colonies were evaluated on the 5th day of every passage.

Characterization of putative spermatogonial stem cells

The alkaline phosphatase (ALP) staining was performed using a commercially available kit (Sigma-Aldrich; Cat#86C), as per the protocol described elsewhere[16] and the manufacturer's instructions. After removal of culture media and thrice washing with DPBS, the cell colonies were fixed with citrate-acetone-formaldehyde solution for 60 s, and three washings were done using deionized water. After that alkaline dye was added and the cultures were incubated for 15 min at RT (25°C). The dishes were rinsed thrice with deionized water and counterstained with neutral red stain for 2 min. The colonies were then washed thoroughly to remove the extra stain. The red coloured colonies were considered positive for ALP activity and were presumed to be pSSCs.

The expression of OCT-4 and PGP-9.5 in pSSC colonies was examined by immunofluorescence staining. The colonies were fixed using 4% paraformaldehyde in DPBS for 30 min at RT before permeabilized by 0.5% Triton X-100 (Sigma-Aldrich; Cat#T8787) in DPBS for 30 min. The cells were then incubated with blocking solution (2% BSA in PBS) for 30 min and primary antibodies i.e., mouse monoclonal PGP-9.5 (1:100; Invitrogen, Cat# 480012) and OCT-4 (1:50; Invitrogen, Cat# MA5-31458) for 1.0 h at RT, followed by three washings with DPBS. Further, the FITC labeled secondary antibody was added (goat anti-mouse IgG; 1:500; Sigma-Aldrich, Cat# F0257) and incubated for 45 min in dark at RT. After counterstaining with DAPI (0.3 μg/mL) for 1 min, cells were washed 5 times with DPBS before examination and imaging under a fluorescence microscope (Nikon, Tokyo, Japan).

Cellular senescence staining

The senescence-associated β-galactosidase (SA-β-gal) staining of pSSC colonies was performed with the senescence-related β-galactosidase staining kit (Merck Millipore, Cat#KAA002), following the manufacturer's guidelines. Briefly, pSSCs cultured on uncoated and 0.01% PDL-coated dishes (Gr. II) at P-7 were rinsed with PBS and fixed with fixing solution for 15 min of incubation at RT. After washing, colonies were incubated with SA-β-gal staining solution for 4 h at 37°C. The number of SA-β-gal positive cells (blue colour), out of a total number of cells, was assessed under a phase-contrast inverted microscope (NIKON, Eclipse, TE 2000U) to evaluate the extent of senescence in different culture conditions[17].

Statistical analysis

The differences among the groups were statistically analyzed using a one-way ANOVA test. The quantitative data are expressed as mean ± SEM. The statistical analysis for senescence detection was performed using the chi-squared test. A P value <0.05 was considered statistical significance.

Results

As protocol involved 2-step 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 while incubation with second enzymatic digestion resulted in the release of most of the spermatogonia from the seminiferous epithelium as single cells.

Isolation and enrichment of caprine pSSCs

Isolation and enrichment of pSSCs were done successfully by a standardized two-step enzymatic digestion method. Differential plating method helped in enriching the SSCs by collecting germ cells, transferring them to new wells subsequently, and helped in eliminating the somatic cells. As per a morphological examination, after 8 days of culturing pSSCs were enriched to about 80%. The SSCs colonies were noticeable on the 8th day and were observed as round or oval bodies, with large nuclei and little cytoplasm. Subsequently, pSSCs formed cell clusters with a confluent monolayer of Sertoli cells. The number of pSSCs in the colony increased followed by an increase in the size of the colonies finally attaining a three-dimensional (3D) rose shape (rosette colony).

Growth kinetics of cultured pSSCs

In this study, distinct morphological patterns of pSSC colonies in uncoated and PDL-coated culture plates were observed (5th day) (Fig. 1 A-D). Total pSSCs colonies in Gr. II at P-2 were significantly higher as compared to Gr. I (3.30-fold; P <0.05), Group III (2.43-fold; P <0.05) and Gr. IV (2.06-fold;

P <0.05). Furthermore, colony number in all groups decreased in the latter passage (P-7). However, in that case, Gr. II had a significantly higher number of colonies as compared to Gr. I (control), Gr. III and Gr. IV 4 (2.54-fold, 2.02-fold and 2.10-fold; *P* <0.05, respectively) (Table 1).

It was observed upon comparing the total number of cluster colonies in different groups (on 5th day) P-2 that, they were significantly (*P* <0.05) higher in treatment groups (Gr. II-IV) than Gr. I (control) (Table 1). Furthermore, in P-3, the number of rosette colonies in Gr. II was significantly (*P* <0.05) higher compared to Gr. I (control), Gr. III and Gr. IV (3.75-fold, 2.14-fold and 1.87-fold, respectively). The total number of rosette colonies in control and different treatment groups are presented in Table 1.

Characterization of pSSCs

Alkaline phosphatase staining showed dark red color in colonies whereas the Sertoli monolayer was lightly colored confirming the presence of undifferentiated cells (Fig. 1 E and F). Furthermore,

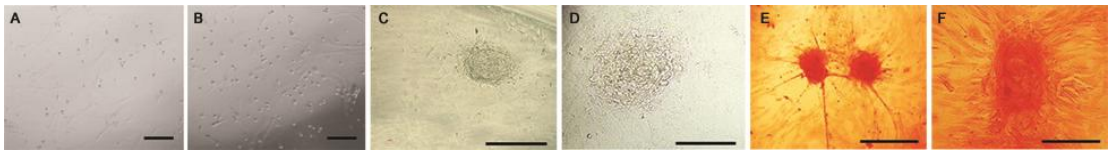

Fig. 1 — (A) pSSCs colonies in uncoated well (passage 1, 100X magnification); (B) pSSCs colonies in PDL coated well (passage 1, 100X magnification); (C) Rosette colony in uncoated well (passage 3, 200X magnification); (D) Rosette colony in PDL coated well (passage 3, 200X magnification); (E) Expression of alkaline phosphatase staining of pSSC colony in uncoated well (passage 3, 200X magnification); and (F) Expression of alkaline phosphatase staining of pSSC colony in PDL coated well (passage 3, 200X magnification). [pSSCs, putative spermatogonial stem cell, Scale bar, 50 µm (A and B) or 100 µm (C – F)]

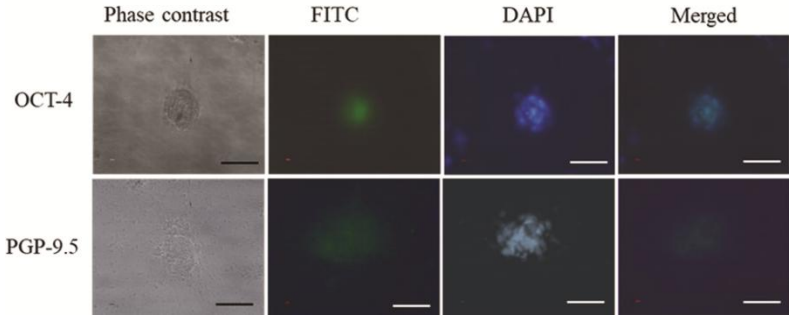

Fig. 2 — Representative images of immunofluorescence staining of cultured putative spermatogonial stem cell (pSSCs) colonies stained for stem cell markers (octamer-binding transcription factor-4, OCT-4 and Protein gene product-9.5, PGP-9.5). [Cells were grown on the 24-well culture plates and stained for markers with specific primary and secondary antibodies followed by counter-stained with DAPI. Scale bar, 50 µm]

Table 1 — Number of the cluster, rosette, and total number of putative spermatogonial stem cell colonies (mean ± SEM) in different groups during different passages (P)

Type of colonies	Groups	Passage						
		P-1	P-2	P-3	P-4	P-5	P-6	P-7
Cluster colonies	Gr. I	3.5±1.5	3.5±0.5^{a}	4.5±0.5	3.5±0.5^{ad}	0.0±0.0	4.5±2.5	4.0±0.0
	Gr. II	8.5±1.5	7.5±0.5^{b}	8.0±0.0	5.5±0.5^{a}	4.0±1.0	4.0±0.0	3.5±2.5
	Gr. III	10.5±1.5	6.5±0.5^{b}	6.5±1.5	1.5±0.5^{dc}	2.0±2.0	5.0±1.0	5.0±2.0
	Gr. IV	9.0±1.0	7.5±0.5^{b}	7.5±1.5	0.0±0.0^{c}	0.0±0.0	4.5±0.5	2.5±0.5
Rosette colonies	Gr. I	0.5±0.5	3.5±0.5^{ab}	2.0±0.0	0.0±0.0^{a}	4.0±1.0	2.0±1.0	3.5±0.5
	Gr. II	4.5±0.5	6.0±1.0^{a}	7.5±0.5^{a}	5.5±0.5^{ac}	6.5±2.5	2.5±0.5	4.0±2.0
	Gr. III	1.5±0.5	1.5±0.5^{b}	3.5±0.5	7.5±0.5^{bc}	16.0±12.0	4.0±0.0	4.5±0.5
	Gr. IV	2.5±1.5	6.5±0.5^{ab}	4.0±0.0	10.0±2.0^{bc}	4.0±2.0	2.0±0.0	2.5±0.5
pSSC colonies	Gr. I	34.0±19.0^{a}	60.0±5.0^{a}	66.5±15.5^{a}	20.5±2.5	4.0±1.0^{a}	6.5±3.5	15.5±2.5^{a}
	Gr. II	249.5±1.50^{bc}	200.0±16.0^{b}	154.0±15.0^{b}	15.5±2.5	17.5±5.5^{ac}	8.5±1.5	39.5±2.5^{b}
	Gr. III	129.5±34.5^{ab}	82.0±3.0^{a}	116.5±1.5^{ab}	18.0±3.0	30.0±5.0^{bc}	10.0±2.0	19.5±2.5^{a}
	Gr. IV	91.5±22.5^{ad}	97.0±5.0^{a}	84.0±9.0^{a}	10.0±2.0	9.0±3.0^{ac}	6.5±0.5	18.0±3.0^{a}

[The number of cluster and rosette colonies were counted on the 5th day of culture during respective passages, The total number of putative spermatogonial stem cells include single, paired, cluster, and rosette colonies. Numbers with different superscripts (a, b, and c) within the same column of the type of colonies, differ significantly (*P* <0.05). Gr. I, control; Gr. II, 0.01% PDL, 5 min; Gr. III, 0.01% PDL, 30 min; and Gr. IV, 0.01% PDL, 60 min]

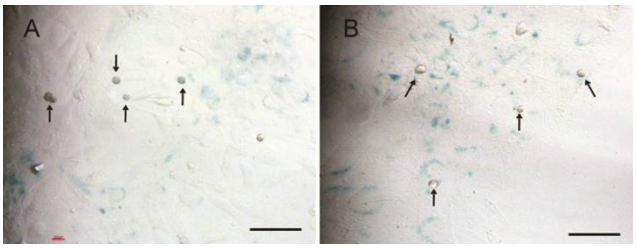

Fig. 3 — (A) Blue colour stained single pSSCs colonies observed in uncoated well after β-galactosidase staining showing senescence (passage 7, 100X magnification) depicted by the arrows; and (B) Unstained single pSSCs colonies observed in PDL coated well showing no senescence (passage 7, 100X magnification) depicted by arrows. [pSSCs, putative spermatogonial stem cell, Scale bar, 50 µm]

Table 2 — Effect of poly-D-lysine (0.01%) on senescence of caprine putative spermatogonial stem cells (pSSCs) in P-7

Groups	No. of stained pSSC colonies	Total pSSC colonies	Senescence
Gr. I	14.0±1.52[a]	18.0±3.24[a]	77.7%
Gr. II	10.0±2.09[b]	26.0±1.13[b]	38.4%

[Numbers with different superscripts (a and b) within the same column differ significantly (P <0.05). Gr. I, control; and Gr. II, 0.01% PDL, 5 min]

the positive signals were observed in the cultured colonies after immunostaining with OCT-4 and PGP-9.5 markers (Fig. 2). However, no difference in characterization of colonies with control and PDL coated groups was observed.

Effect of PDL on senescence

In our study, the proliferation capacity of pSSCs improved with PDL coating, as presented by the β-galactosidase staining (Fig. 3). The percentage of senescence without PDL coating was significantly (P <0.05) higher than the cells with PDL-coated plates (Table 2). In Gr. II, staining was observed in rosette and cluster colonies with no staining observed in single colonies while in the control group rosette, cluster, and even single colonies were stained. This indicates the presence of senescence in cells even at an early stage (single colonies) of P-7 in uncoated wells.

Discussion

We established an easy, simple and efficient culture system for obtaining highly enriched populations of caprine SSCs. The SSCs hold significant promise for future use in clinical applications like preservation of male germ cells for breeding program[18] through transplantation into the suitable recipient animals[5]. As SSCs are sensitive to external stimuli and promptly enter a condition of senescence, isolation, culture, and expansion from essential source is very critical for their clinical application. In general, during *in vitro* culture, cells are exposed to a complex and highly structured microenvironment that is regulated by different biophysical, biochemical factors, and ECM *in vivo*[19]. The proteins of ECM comprise a microenvironment that gives structural support and attachment to the cells[20]. Therefore, the application of suitable ECM in *in vitro* culture conditions has a significant impact on the fate of SSCs[21]. This study highlights the fascinating probability that PDL could provide a favourable environment for SSC culture *in vitro*.

In our study, caprine pSSCs were obtained effectively from pre-pubertal goat testis by a two-step enzymatic digestion and enriched by differential plating method. Furthermore, with a different method of adherence, fibroblasts, and other cells started adhering within four hours after seeding. Moreover, based on the cluster or larger size colonies, the culture after differential adherence was found to be better than primary culture (P-0). This could be due to fact that this procedure helped in enriching the SSCs by collecting the floating germ cells and transferring them to new plates subsequently, in turn eliminating somatic cells. Similarly, the method of differential platting is used for recovery of the higher number of viable SSCs from testicular cell suspension of sheep[22] and goat[23]. Therefore, with the help of two-step enzymatic digestion and differential platting methods, the testis cell population with a high proportion of pSSCs was identified, cultured, and further passaged till the 7th passage.

As hypothesized, we observed an increase in the number of pSSCs cultured on PDL-coated plates in comparison to uncoated plates. Also, with 0.01% PDL coating, the diameter of colonies was larger and colonies were mostly single, circle to oval in shape in the early stages of passages[15]. With time, the colonies were paired and cluster forms, finally attaining rosette shape in both control and PDL coating groups. On comparing, coating method of 0.01% PDL with different time interims, the best results for *in vitro* colonization and proliferation of caprine SSCs were observed with 0.01% PDL with 5 min coating incubation (Gr. II). Moreover, our results suggest that the effect of PDL decreased as the coating time increased indicating that a longer period of PDL exposure did not give an advantage in terms of cellular attachment. Therefore, it can be suggested

that the longer coating time of PDL does not provide the appropriate environment required to produce the needed extracellular matrix and indeed inhibited the cell attachment to the extracellular matrix. However, further investigation is needed to establish more clearly.

Sometimes, the colonies were paired and cluster forms in both control and PDL coating groups, and especially a greater number of pSSCs colonies in cluster form observed at P-2 and in rosette form were observed at P-3 in PDL coating (Gr. II). We have observed that PDL helped in faster conversion of cluster colonies into rosette colonies thus demonstrating the positive effects of PDL on cell attachment, proliferation, and expansion of pSSCs *in vitro*. The Sertoli cells potentiate the *in vitro* proliferation of co-cultured stem cells by their growth factors and cytokines[24]. Therefore, the indirect effect of PDL on the proliferation of SSCs through activities of Sertoli cells warrants further investigations.

The SSCs normally dwell in a particular niche *in vivo*, which predominantly comprises the ECM. This ECM provides structural and biochemical support to the cells and has different capacities including cell adhesion[25], cell to cell communication, and differentiation[26]. We observed a higher proliferation of pSSCs in the PDL-coated plates compared to the uncoated plates. These results are in agreement with the earlier reports suggesting improved functional characteristics of human pluripotent stem cells grown onto the poly-lysine-coated dishes[27] and goat male germ cells in the presence of different ECM proteins[20]. In contrast to this, the beneficial effect of PDL on short-term maintenance, proliferation, and colony formation was not observed in porcine gonocytes[28]. As a non-specific attachment factor, PDL enhances the cell adhesion property because of its strong affinity for proteins and electrostatic interactions between the positive charges on the PDL molecule and the negative charges present on the cell membrane[29,30]. This strategy could be useful for *in vitro* expansion of highly functional SSCs and may be useful for cell-based therapeutic applications.

Strong ALP activity was observed in pSSCs indicating high expression of alkaline phosphatase with dark pink colour whereas the feeder cells were lightly coloured. SSCs were also positive for the transcription factor OCT-4 and PGP-9.5 which was confirmed by intra-nuclear green fluorescence. Our outcomes were positive for ALP, OCT-4 and PGP-9.5 staining on both the uncoated and PDL coated plates[31-33]. The OCT-4 expression indicated that the SSC colonies were pluripotent and in the undifferentiated stage as reported earlier (Wang *et al.*[34] in goat, Kala *et al.*[31] in buffalo, Koruji *et al.*[35] in mouse, Zhao *et al.*[36] in pig and Liu *et al.*[37] in human). Furthermore, it is observed that OCT-4 maintains self-renewal and prevents senescence in mesenchymal stem cells through the downregulation of p21 by DNA methyltransferases[38]. Our results about the presence of PGP-9.5 were in the agreement with Ebata *et al.*[39] (mouse), Park *et al.*[40] (pig), Sharma *et al.*[41] (goat), Izadyar *et al.*[42] (bovine), Choi *et al.* (donkey)[43], and Ebata *et al.*[39] (human). The PGP-9.5, also known as UCHL-1, is the specific marker for spermatogonia and is conserved in the mammalian testis[44] including goats[45]

In addition, PDL as a coating substrate delayed senescence of SSCs which were attached on PDL through the cell to ECM interaction, demonstrating that PDL-coated plates provide the necessary microenvironment for optimal growth of SSCs *in-vitro*. Cells retained proliferation with delayed senescence on PDL than the control group. PDL used in our study hindered pSSCs senescence, as determined by -galactosidase staining. Numbers of stained pSSCs colonies were significantly (P <0.05) higher in control than the PDL coating (Gr. II) at P-7, showing that PDL-coated plates provided the necessary microenvironment for optimal growth of caprine pSSCs *in vitro*[17]. The upregulation of stemness factors may play a crucial role in increased proliferation and delaying replicative senescence.

Conclusion

The pSSCs grown onto the PDL-coated plates yield more number of larger colonies and lower expression of β-galactosidase indicating improved proliferation rate and lower senescence of pSSCs on PDL-coated plates than uncoated plates. Thus, the coating of the culture plate with PDL provides a favorable microenvironment for functional characteristics of cultured pSSCs. The findings of the present study may provide important information for the design of biomaterials and will contribute greatly to the improvement of the SSC culture system *in vitro.* However, our results require further studies with

regards to the specific genes that have functional implications on *in vitro* proliferation and senescence of pSSCs.

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Conflict of Interest

Authors declare no competing interests.

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