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STEM CELL BIOLOGY

Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers

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Abstract
Introduction Presently the techniques for making transgenic animals are cumbersome, required costly instruments and trained man-power. The ability of spermatogonial stem cells (SSCs) to integrate foreign genes has provided the opportunity for developing alternate methods for generation of transgenic animals. One of the big challenges in this field is development of the methods to identify and purify donor SSCs by antibody mediated cell sorting.
Purpose The present study was aimed to identify goat subpopulations of SSCs using polyclonal antibodies against PGP9.5 and c-kit molecular markers as well as the growth characteristics of SSCs during short term culture.
Methods One month old goats' testicular samples were subjected for immunohistochemical and immunocytochemical evaluations. The enzymatically isolated SSCs were cultured in DMEM plus FCS supplemented with (treatment) or without (control) growth factors (GDNF, LIF, FGF, and EGF) for 2 weeks. At the end of culture the morphological characteristics of SSCs colonies and immunocytochemical staining were evaluated.
Results The number and size of colonies in treatment groups were significantly ($P<0.01$) higher than corresponding values in controls. The presence of PGP 9.5 and c-kit antigens was confirmed in immunocytochemical evaluation. In immunocytochemical evaluation, the proportion of c-kit and PGP9.5 positive cells were significantly ($P<0.001$) higher in control and treatment groups, respectively.
Conclusions The presence of PGP9.5 and c-kit antigens was confirmed in goat SSCs. Moreover, culture medium supplementation with growth factors could effectively retain the undifferentiation status of SSCs, reflected as a higher population of PGP9.5 positive cells, after short term culture.

Keywords PGP9.5 · c-kit · Goat · Spermatogonia · Growth factors

Capsule This study confirmed the different expression of PGP9.5 and c-kit markers in subpopulation of undifferentiated and differentiated goat type A spermatogonia.

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Introduction

Spermatogenesis is a complex process comprising proliferation, differentiation, and maturation of many different subsequent cell types [1] resulting in production of unlimited numbers of spermatozoa throughout the adult life of the male [2, 3]. Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and comprising only 0.03% of all germ cells in testes [4]. They are defined like all other stem cells by their ability to balance self-renewing and at the same time to generate the cascade of differentiating germ cells that will eventually lead to the formation of sperm [1, 5–8]. Among stem cells in a male individual, SSCs are unique cells in an adult body that divide mitotically and contribute genes to subsequent generations making them a perfect target for genetic manipulations [3].

Experiments using spermatogonial transplantation in rodents have shown the technique to be an important new tool for studying spermatogenesis in mammals and have opened the possibility of using SSCs in domestic animals for preservation of fertility, dissemination of genetics in animal production, and generation of transgenic animals.

It would be a great advantage if various developmental steps carried out by germ cells could be studied in vitro. To study the biochemical and colonogenic characteristics of SSCs, large enough populations of SSCs must be isolated. One way to reach this goal is to propagate these cells in vitro and various attempts to achieve this have been carried out [9, 10]. Kanatsu-Shinohara et al. [11] by using a combination of growth factors and a special stem cell medium, by way of continuous subculturing during 4–5 months, could achieve an expansion of SSCs in the order of 10-12-fold.

In order to isolation and purification of spermatogonia, the availability of markers that can conclusively establish the identity of the spermatogonia is essential. One such a marker is c-kit, the receptor for stem cell factor (SCF), which is expressed by some Aal, A1–A4, In, and B spermatogonia [12]. The presence of c-kit receptor in SSCs, however, has been a matter of debate for many years [13, 14].

Another molecular marker for identification and isolation of spermatogonia is protein gene product 9.5 (PGP9.5; ubiquitin Cterminal hydrolase L-1) that is originally isolated as a neuron-specific protein. Antibody against this protein has been used for immunohistochemical detection of mouse [15], bovine [16], ram [14], human [17] and porcine [18] spermatogonia. Respecting to the lack of information on goat type A spermatogonial markers and the inadequate information regarding to the isolation, identification, and culture of goat SSCs, and considering the application of transfected SSCs as an efficient tool in production of transgenic animals, the present study was aimed to investigate the identification of prepubertal goat SSCs using polyclonal antibodies against PGP9.5 and c-kit molecular markers. The effect of type of enzyme (collagenase I and collagenase IV) on the speed of tissue digestion as well as colonogenic characteristics of goat SSCs was compared.

Materials and methods

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

Histological evaluation of testes

Following castration of 1 month old goat, the testes transported to the lab in transition media (PBS+antibiotics) at 37 °C. After macroscopic evaluation of the testes for any pathologic signs (trauma, cyst, tumor and hematoma), the testes were washed (3 times) with transition media and their weigh, dimensions, and volume were then measured. The testicular volume (TV) determined according to the formula described by Steger and Wrobel (1994): TV=1/6pa2bk (cm3), where: a = width, b = length and k=0.945. The tunica albuginea was removed under sterile condition and the testes minced into small pieces using fine scissors.

For histological evaluation a small sample of testes fixed overnight in Bouin's solution, washed in 70 % ethanol, embedded in paraffin and sectioned at 5 μm using standard procedures. Subsequent sections stained with Hematoxylin and Eosin and examined under a light microscope for identification of cell types and their developmental stages. The diameter of semniferous tubules measured and the number of sertoli cells, large and small spermatogonia counted in 110 tubules.

Isolation of spermatogonia

The samples were prepared using a protocol previously described by Izadyar et al. (2002) with minor modifications. Briefly, the testes samples washed several times with transition media and transferred into the Dulbeco Modified Essential Medium (DMEM) supplemented with $NaHCO_3$ (14 mol/l), Hepes (15 mol/l), NEAA (10 μl/ml), penicillin (50 IU/ml) and streptomycin (50 mg/ml) for 5-8 min. The SSCs were isolated through two step digestion methods. During enzymatic digestion two types of collagenases (I and IV) were compared. The samples incubated in DMEM containing either 1 mg/mL collagenase type I (C0130) or 1 mg/mL collagenase type IV (C1889) at 38 °C, 5 % CO_2 for 60 min. The samples examined with an interval of 1 min for determination of disentanglement. After initiation of tissue disentanglement the somniferous tubules' fragments were separated from interstitial cells by centrifugation at 100 x g for 1 min, and the supernatant, containing mostly the interstitial cells, was discarded. The tubule fragments washed two times with ice-cold DMEM. In a second digestion step, the tubules' fragments were incubated in trypsin/EDTA (0.25 %/1 mM) for 12-25 min. Undigested debris removed by centrifugation at 100 x g for 1 min. The supernatant was then processed by sequential filtration through 60 μm nylon mesh (Small part, F062N-08-C). The filtrate was centrifuged at 500 x g for 5 min, and the pellet was resuspended in DMEM supplemented with 10%FBS (Gibco) and NEAA. In the final cell suspension the different testicular cell types including sertoli cells and spermatogonia with different sizes were determined using light microscope. Total cell number and cell viability were determined after Trypan Blue staining. The cell suspension was mixed with 0.4 % Trypan Blue (1:1, v/v). The number of live (Trypan Blue excluding) and dead cells were determined using hemocytometer.

Cell culture

The cells were cultured in 12-well chamber slides (Falcon, USA) in DMEM supplemented with either 10 % FBS

(Control group) or 10 % FBS plus 20 ng/ml EGF, 10 ng/ml bFGF2, 100 ng/ml LIF, and 40 ng/ml GDNF (treatment group) at 38 °C in a humidified atmosphere with 5 % CO_2 for 2 weeks. The medium was refreshed once a week and the number of SSCs colonies and their morphological characteristics (size and shape) were then evaluated at the end of each week. The immunocytochemical staining for evaluation of PGP9.5 and c-kit markers was carried out on prepared cytospin slides prepared from colonies collected at day 14.

Immunohistochemical localization of PGP9.5 and c-kit

Type A spermatogonia was identified through immunohistochemical staining, as described by Rodriguez-Sosa et al. (2006) with minor modification. Briefly, testicular samples used for immunohistochemical staining were fixed overnight at 4 °C in Bouin's solution. They were subsequently washed in 70 % ethanol, embedded in paraffin and sectioned at 5 μm using standard procedures and mounted on slides. The prepared slides were fixed in cold neutral buffered formalin (NBF) for 5 min, washed (three times, 4 min each) by Tris–buffered saline containing 1 % bovine serum albumin (TBS/BSA) and processed immediately for immunostaining.

Antigen retrieval was performed on sections through immunostaining for PGP 9.5 and c-kit. The slides were placed in citrate buffer (0.01 M, pH 6.0), boiled for 8 min at high power in microwave oven, and rinsed in TBS/BSA. All sections were exposed to 0.3 % H2O2 for 15 min in dark to inhibit endogenous peroxidase and washed in TBS/BSA (three times, 4 min each). The first unspecific site blocking was done with avidin/biotin. Therefore, the slides were incubated with avidin for 10 min in dark, and after three times washing again incubated with biotin for 10 min in the same condition. Another unspecific site blocking was done with 5 % sheep serum in PBS for 10 min at room temperature. Subsequently, the slides were incubated with unconjugated primary antibodies including rabbit anti-PGP 9.5 (Dako, Carpinteria, CA, USA) and rabbit anti c-kit (Santa Cruz, Santa Cruz, CA, USA), each used at 1:400 in PBS with 2.5 % goat serum (PBS-GS), for 1 h at room temperature. After washing three times in PBS (5 min each), the sections were exposed to secondary antibody (biotinylated sheep anti-rabbit IgG, Avicenna Research Institute, Iran) for 45 min at room temperature and washed in TBS/BSA as above. The sections were exposed to diluted HRP-conjugated streptavidin (Biosource, USA), 1:250 (v/v), for 30 min and then washed with TBS/BSA. At the final step, color was developed by the addition of 3, 3'- Diaminobenzidine (DAB; Roche, Germany) for 10 min. The slides were rinsed thoroughly in distilled water, counterstained with Harris hematoxylin for 30s, washed in distilled water, dehydrated in graded alcohols, cleared in xylol, and were then mounted in Entellan (Merck, Germany).

Immunocytochemistry

The testicular cells were tripsinized after 2 weeks culture, centrifuged at 500 x g in 4 °C for 5 min, washed three times with PBS, placed 4×10^4 cells in each slide, centrifuged onto slide at 400 rpm (Cytospin) for 5 min, fixed in acetone for 90 min in -70 °C and were then placed in 4 °C for 90 min. After drying the slides, cells immunostained as described above except that the washing was done by Tween 20 (0.2 % in PBS). The slides were mounted by glycerol/PBS (50/50). The percentages of PGP9.5 and c-kit positive and negative cells were evaluated by cell counting of prepared immunocytochemical slides.

Results

Pathology and histology evaluations

No sign of physical and pathological abnormalities were detected in testes. The testicular volumes were 2.5 cm^3 and 2.2 cm^3 for testes 1 and 2, respectively. In histological evaluation, the immature sertoli cells, spermatogonial cells, and a few number of leydig cells were identified by morphological evaluations (Fig. 1a, b). The most germ cells at this stage were type A spermatogonia. The somniferous tubules had no lumen and the different subpopulations of type A spermatogonia were mostly localized in the middle of the somniferous tubules (Fig. 1a, b).

In histological evaluations of seminiferous tubules, the presence of three groups of spermatogonia with different sizes was determined (Fig. 1a, b). The proportion of sertoli cells in seminiferous tubules >80 μm in diameter was higher than those with diameter ≤80 μm. In both categories of seminiferous tubules the proportion of sertoli cells was greater than large and small spermatogonia ($P<0.05$). The proportion of large spermatogonia in seminiferous tubules >80 μm diameter was higher than small spermatogonia ($P<0.05$, Table 1).

Validation of germ cell markers by immunohistochemistry

The primary polyclonal antibodies against PGP 9.5 and c-kit antigens could identify the presence of germ cells in 1 month old goat testes. In cross-sectional imaging, the PGP 9.5 positive cells were comprised of basal (As,

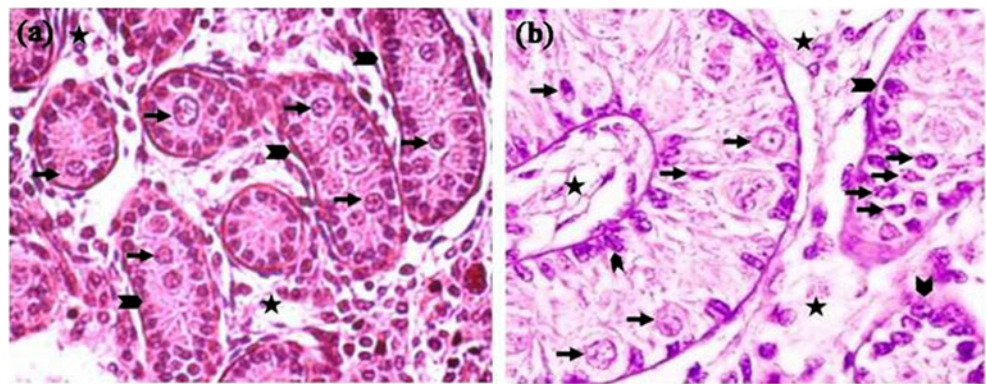

Fig. 1 Histological sections of 1 month old goat testes stained with Hematoxylin and Eosin. Spermatogonial cells (*arrows*, **a**, **b**), immature sertoli cells (*arrowheads*, **a**, **b**) and a few number of leydig cells (*asterisk*, **a**, **b**) have been shown. The different sizes of spermatogonia and the absence of central lumen are noticeable

Ap) and some aggregated spermatogonia (Aal) which positioned in closed contact with each other (Fig. 2a, b). The c-kit positive cells were clearly a different population of germ cells and were comprised of some aggregated and committed type A spermatogonia. There was a difference in intensity of c-kit immunostaining between type A spermatogonia as such the majority of aggregated type A spermatogonia had a stronger c-kit immunoreactivity than committed type A spermatogonia (Fig. 3a, b).

In testicular sections, immunoreactivity was strongly detectable in the cytoplasm of gonocytes (Fig. 4a). The basal stem cells (small round cells) with a spherical nucleus containing one to three centrally irregular dense nucleoli (1–3 μm in diameter) were comparable to As and Apr spermatogonia (Fig. 4b). Aggregated spermatogonia with different sizes from small to large, containing one to two nucleoli were resembled to the Aal spermatogonia (Fig. 4c). Committed spermatogonia, the largest type of spermatogonia found in the testis with a large centrally located nucleolus was comparable to A1–A4 differentiating spermatogonia (Fig. 4d).

Table 1 Proportion of different cell types (Mean±SEM) in 1 month old goat somniferous tubules

Cell type	Semniferous diameter (μm)	
	≤80 n (%)	>80 n (%)
Sertoli cell	8.4±0.4 (69.9±1.8)^{a,A}	9.8±0.4 (73.5±1.6)^{b,A}
Large spermatogonia	1.9±0.2 (16.8±1.6)^{B}	2.0±0.1 (15.9±1.2)^{B}
Small spermatogonia	1.6±0.1 (13.4±1.0)^{B}	1.4±0.1 (10.6±1)^{C}

^{a,b} Numbers with different superscript letters in the same row differ significantly ($P<0.001$)

^{A,B,C} Numbers with different superscript letters in the same column differ significantly ($P<0.05$)

Enzymatic digestions

Prior to cell culture, the cells should be isolated from a tissue sample and dispersed as single cells. The speed of testis digestion using collagenase IV was faster ($P<0.001$) than collagenase I (Table 2). No significant difference was observed in cell viability (100 % for both enzymatic digestions) and cell count using two different enzymes.

Colony formation

All cultures started with a mixed population of cells including spermatogonia and somatic cells. At the onset of culture, spermatogonia were identified as round cells with a high nucleus:cytoplasm ratio and spherical nucleus containing one to three dense nucleoli and many cytoplasmic inclusions mostly concentrated at one side of the cell. After 1 week culture, the spermatogonia were observed in different forms as single (Fig. 5a), paired (Fig. 5b, c), aligned and cluster (Fig. 5d) forms.

After a few days, the somatic cells were constituted a confluent monolayer as a feeder layer with grown spermatogonia at its surface.

The colony-forming cells in the initial suspension, including germ cells, were approached each other and became tightly packed as the colonies continued to grow during their development. The SSCs colonies were then formed locally in some areas of the culture plate. As development was progressed, the colonies retained a short physical connection to the underlying monolayer by a stalk (Fig 6a). The colonies were mostly single, circle to oval and biconvex (Fig. 6a, b). Sometimes, the colonies were paired (Fig. 6c), catenarian (Fig. 6d), and cluster forms (Fig. 6e), especially in treatment group, and sometimes as a rosette form in control group (Fig. 6f). Gradually during the first and second weeks of culture, the number of spermatogonia and, therefore, the size of colonies were increased. There was no significant difference between colony number at the end of first and second weeks of culture in both control and growth factors

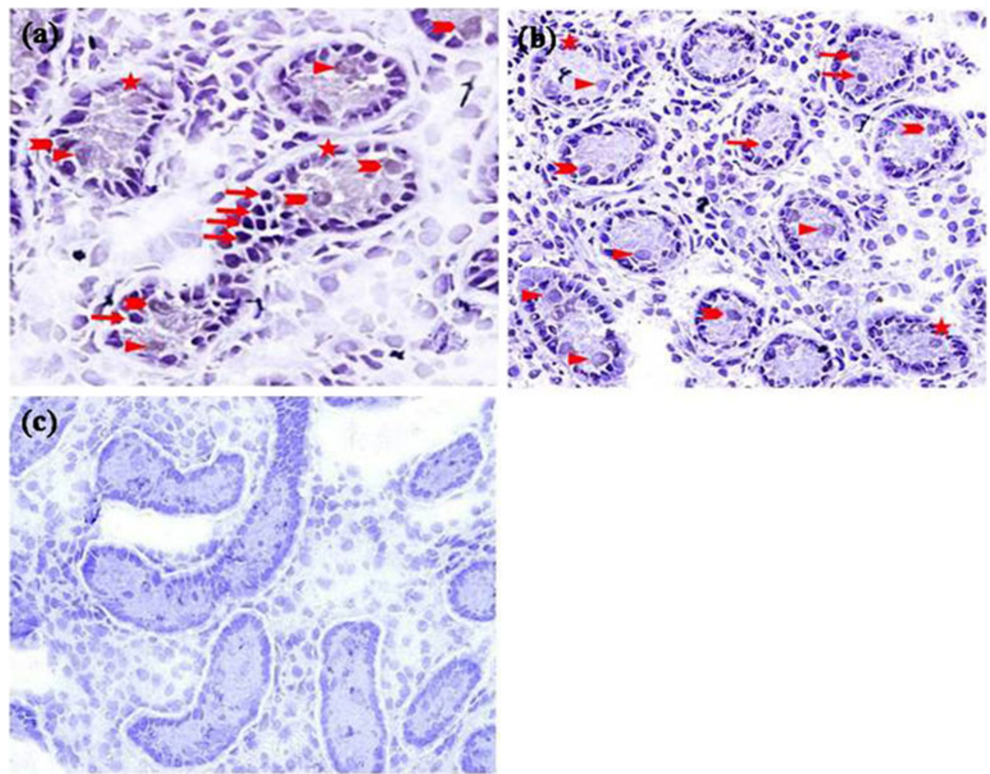

Fig. 2 Immunohistochemical localization of spermatogonia using an antibody against PGP9.5. Both basal (*arrow*, **a**, **b**) and some aggregated (*arrowhead*, **a**, **b**) type A spermatogonia,which positioned in direct contact with each other, were PGP9.5 positive. Note the strong and weak PGP9.5 immunoreactivity was observed among basal (*arrow*, **a**, **b**) and some aggregated spermatogonia (*arrowhead*, **a**, **b**), respectively. Committed spermatogonia (*triangle*, **a**, **b**) and surrounding sertoli cells (*asterisk*, **a**, **b**) were negative for PGP9.5. The Fig 2c served as a negative control for PGP9.5

supplemented groups (treatment group). The colony numbers in treatment group, however, was significantly higher than control at the end of first and second weeks of culture (Table 3).

Concerning to the size of colonies, as shown, the number of middle and large colonies in treatment group at the end of both first and second weeks of cultures were significantly ($P<0.01$)

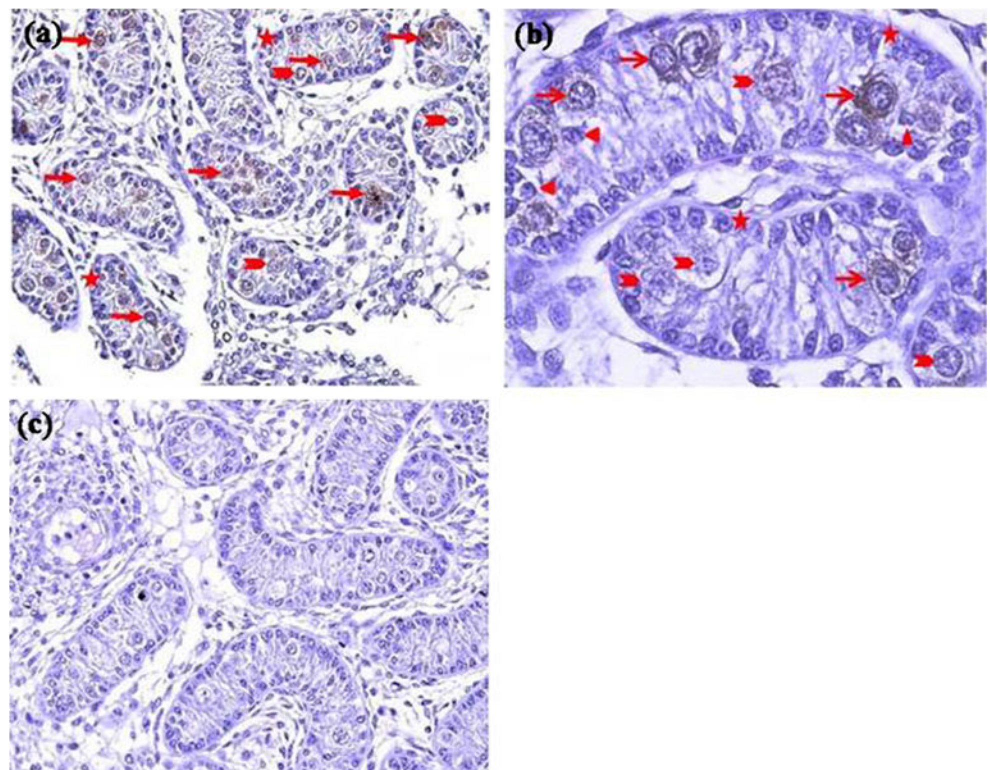

Fig. 3 The difference in the intensity of c-kit staining among spermatogonia. Strong and weak c-kit immunoreactivity was observed among some aggregated (*arrow*, **a**, **b**) and committed spermatogonia (*arrowhead*, **a, b), respectively. Basal spermatogonia (*triangle*, b) and surrounding sertoli cells (*asterisk*, a, b) were negative for c-kit. The fig3c served as a negative control for c-kit**

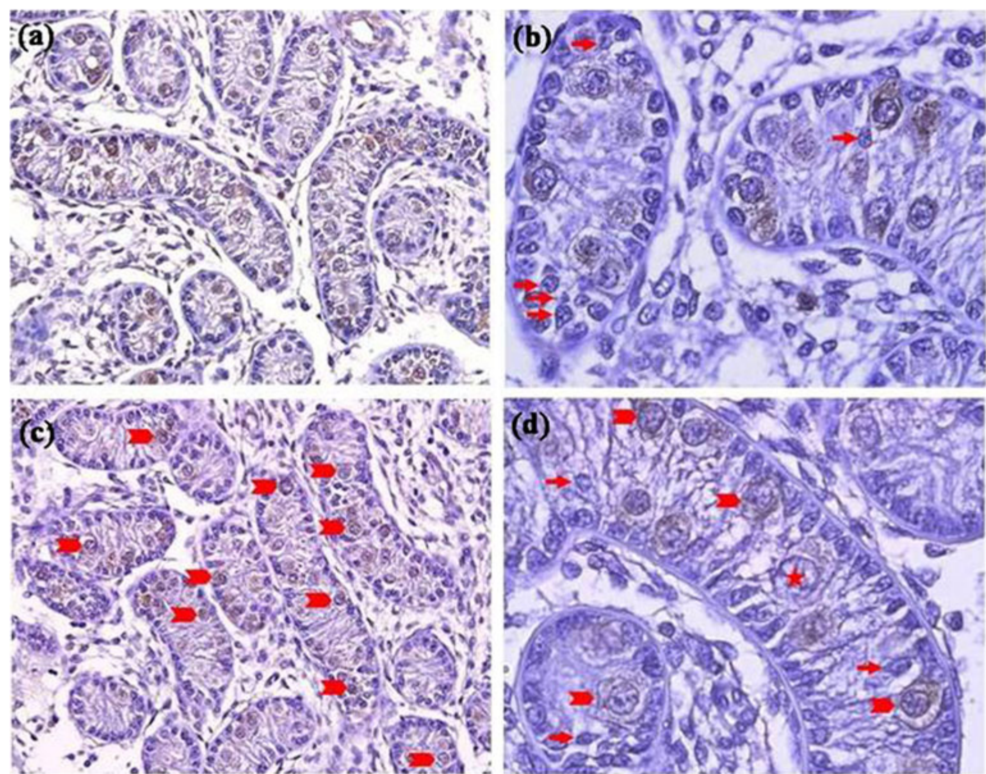

Fig. 4 The immunohistochemical identification of type A spermatogonia using an antibody against c-kit. Immunoreactivity was strongly identified in the cytoplasm of gonocytes (**a**). Three groups of spermatogonia with different sizes were determined. Basal spermatogonia (*arrows*, **b**, **d**) were negative for c-kit. Aggregated (*arrowhead*, **c**, **d**) and Committed (*asterisk*, **d**) spermatogonia were c-kit positive

higher than corresponding values in the same sized colonies in control group (Table 3).

Identification of spermatogonia after culture

After 2 weeks culture, the cells in control and growth factors supplemented groups were trypsinised and mounted for immunocytochemical staining. The proportion of c-kit and PGP9.5 positive cells was significantly ($P<0.001$) higher in control and treatment groups, respectively (Table 4, Fig. 7a-d).

Discussion

The ability to culture male germ-line stem cells in vitro would allow us to unravel the molecular mechanisms driving spermatogenesis, to provide the opportunity for their transfection as an alternative approach in production of transgenic animals, and to characterize the cell signaling pathways inducing stem cell differentiation versus self-renewal [19].

Three types of spermatogonia (types A, Intermediate, and B) were initially described based on their nuclear morphology [20, 21]. In present study, in histological examination of 1 month-old goat testicular sections, two different subpopulations of type A spermatogonia were identified: large cells with a large central nucleolus and small cells with one to three nucleoli (Fig. 1a, b). The difference in size of the spermatogonia might be related to either the phase of cell cycle [22] or the differentiation status of the cells [13].

In morphologic evaluation based on light microscopy and immunostaining, the presence of three groups of spermatogonia with different sizes in seminiferous tubules: basal, aggregated and committed spermatogonia were detected (Figs. 1a, b; 4a-d).

The characteristics of small spermatogonia in the current study were comparable to the basal stem cells (As and Apr spermatogonia), the small round cells with one to three irregular nucleoli. Aggregated spermatogonia with different sizes from small to large, containing one to two nucleoli, were identical to type A aligned spermatogonia (Aal spermatogonia). The subpopulation of large spermatogonia were comparable to committed spermatogonia (A1–A4 differentiating spermatogonia), the largest type of spermatogonia found in the testis with a large centrally located nucleolus.

Table 2 Effect of two enzymes on digestion of goat testis and cell viability (Mean±SEM)

Enzyme	Speed of tissue digestio (min)	Cell viability (%)
Collagenase I	23.7 ± 0.1^{a}	100
Collagenase IV	12.3 ± 0.2^{b}	100

[a,b] Numbers with different superscript letters in the same column differ significantly ($P<0.001$)

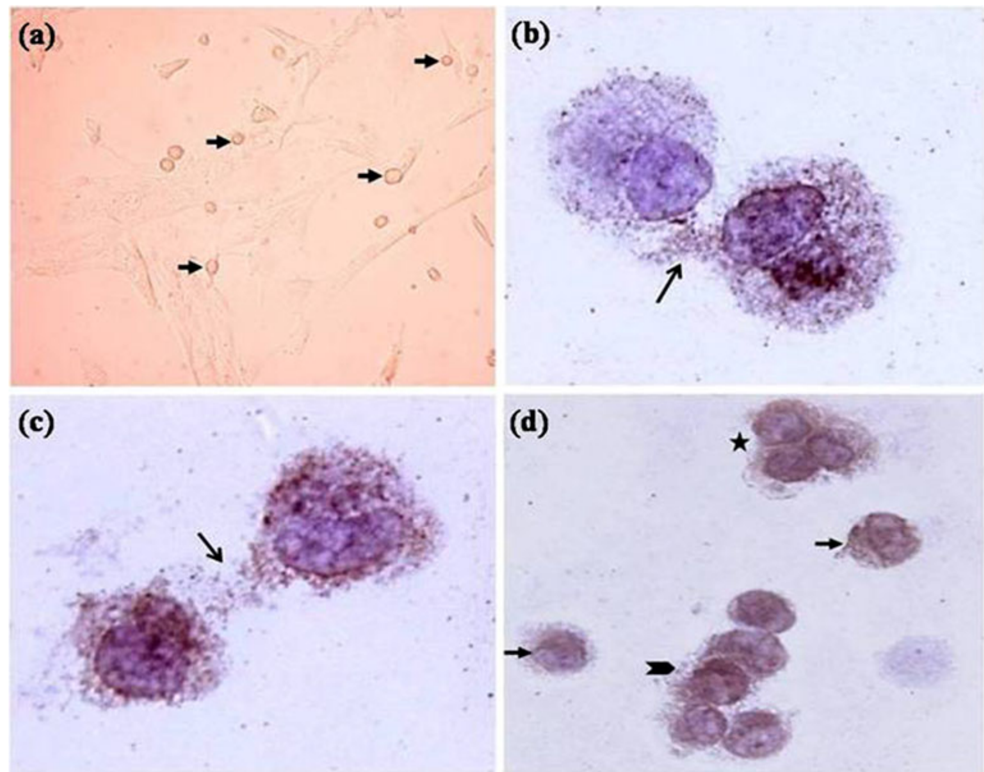

Fig. 5 The spermatogonial stem cells after 1 week culture. After 1 week culture, the spermatogonia were seen to different forms: single (*arrow*, **a**), paired (**b**, **c**), aligned (*arrowhead*, **d**) and cluster (*asterisk*, **d**). At the onset of culture, spermatogonia were round cells with a spherical nucleus, a high nucleus: cytoplasm ratio, and many cytoplasmic inclusions, mostly concentrated at one side of the cell (**c**). The cytoplasmic bridge formed between spermatogonia during colony formation (*arrow*, **b**, **c**)

Therefore, the largest spermatogonia observed in the present study were mostly consisted of subpopulations of differentiating (A1–A4) spermatogonia [13].

In studies on spermatogonial isolation and purification, the availability of markers that can conclusively establish the identity of the spermatogonia is essential. One such a marker is c-kit, the receptor for stem cell factor (SCF), which is expressed by some Aal, A1–A4, Intermediate and B spermatogonia, but not by As and Apr spermatogonia [12]. Therefore, application of c-kit for isolation of spermatogonia is mainly resulted in the selection of more differentiated spermatogonia than undifferentiated SSCs [23]. In the current study nearly all large type A spermatogonia in the immature goat testes were c-kit-positive, while the subpopulations of small (basal) spermatogonia were c-kit-negative. This finding confirmed the classification of Wrobel et al. (1995) in which the smaller spermatogonia were less differentiated than larger ones [24]. In this agreement in mice there is little expression of the c-kit mRNA and protein in undifferentiated type A spermatogonia and its expression is clearly enhanced upon differentiation of these cells into type A1 spermatogonia. Therefore, some of the Aal spermatogonia, which are ready to differentiate into A1 spermatogonia, already express some c-kit receptor and that the majority of unstained A spermatogonia are likely the remaining undifferentiated A spermatogonia (i.e. As, Apr and probably some Aal spermatogonia) [12].

A different size of SSCs in the current study was noticed in both c-kit positive and c-kit negative type A spermatogonia, though the c-kit positive cells were larger. Moreover, the cells in groups of A spermatogonia, being Aal spermatogonia, were always large, while the single cells in the sections, being either As or Apr, were often small. It seems during differentiation of Aal spermatogonia which is concurrent with acquiring the c-kit receptor; the cells will grow up in size. This finding was comparable to the Kluin and de Rooij (1981). Therefore, in current study the c-kit receptor was generally expressed in late stage type A spermatogonia including some aggregated, Aal, and committed spermatogonia and not in As and Apr spermatogonia.

Antibodies against PGP9.5 have been used for immunohistochemical detection of SSCs in several species such as: mouse [15], bovine [16], ram [14], humane [17] porcine [18]. This protein is readily observed in both the cytoplasm and nucleus of gonocytes and spermatogonia in bovine testes. In contrast, its signal is relatively faint in cells along the inside of the basement membrane of the mouse seminiferous tubule epithelium. Sertoli cells and spermatogonia also are exhibited weak positive staining for PGP9.5 [18].

The PGP 9.5 positive cells are clearly a different population of germ cells from the c-kit positive cells at this stage of development [14]. These round cells with a spherical nucleus were often arranged in singles or pairs and occasionally observed in the centre of the tubules (mostly 3–4

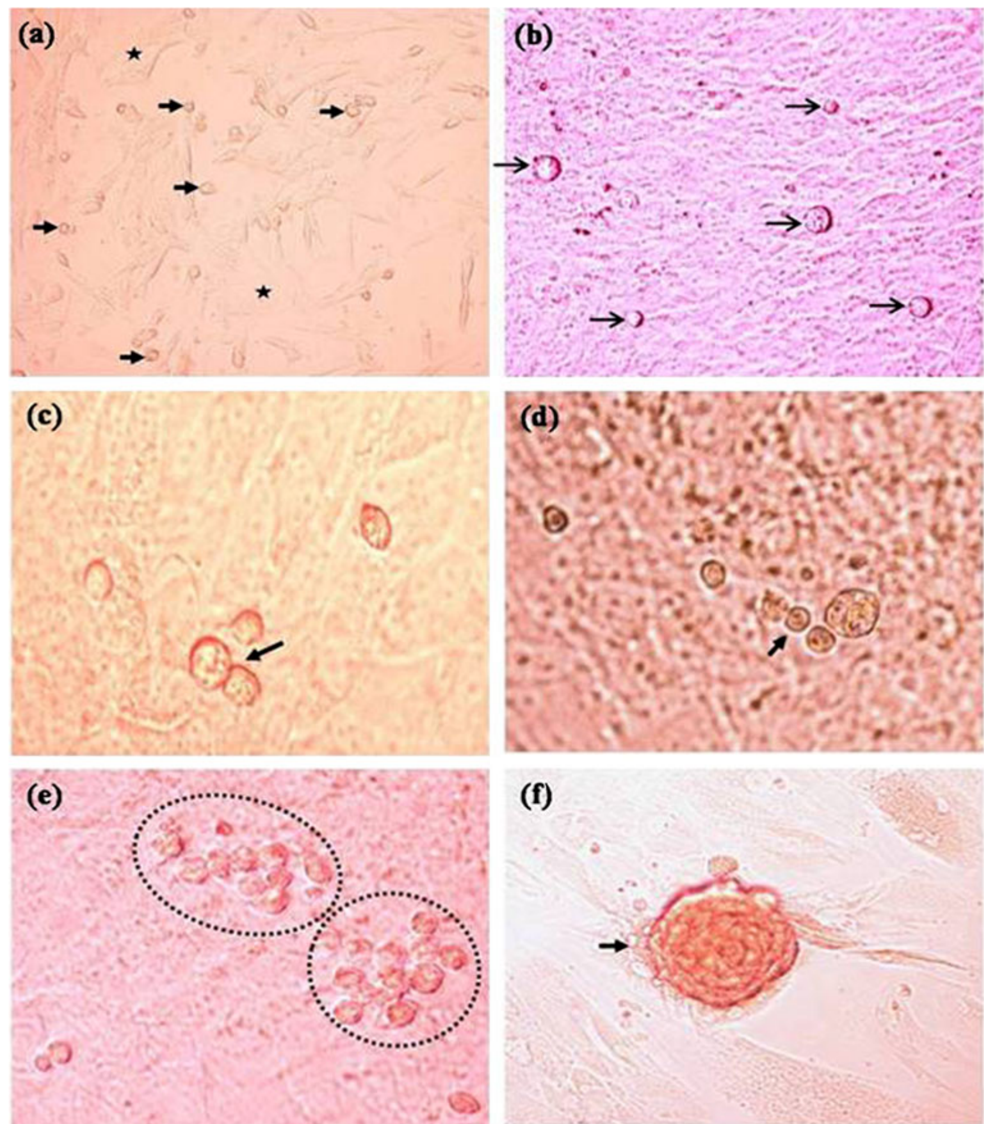

Fig. 6 The SSCs colonies after one and 2 weeks culture. The colonies after 1 week; Sertoli and leydig cells formed the feeder layer (*asterisk*, **a**) with the grown colonies at the surface (*arrow*, **a**). The colonies after 2 weeks were observed in different sizes and forms including: single (*arrow*, **a**, **b**), paired (*arrow*, **c**), aligned (*arrow*, **d**), cluster (*outlined*, **e**) and rosette form (*arrow*, **f**) colonies

PGP 9.5 positive cells per tubule section) including those were identified as As, Ap and some Aal by morphology [14, 25]. Luo et al. (2006) established an enrichment method for spermatogonial cells using PGP9.5 marker. Enriched cells containing SSCs were maintained in culture for 2 weeks without losing their PGP9.5 expression [26]. However, the successfully isolated SSCs in minipigs could not be maintained in culture for more than 10 days [27].

Table 3 Effect of growth factors on number and size of SSCs colonies (Mean±SEM) after short term culture

Experimental Groups	Colony No.		Colony size (μm)							
	One week	Two week	<20		≥20-40≤		40<		Total	
			One week N	Two week n	One week n	Two week n	One week n	Two week n	One week	Two week
Control	47.7 ± 9.0^{A}	53.2 ± 7.5^{A}	$37.29.5^{a}$	27.2 ± 9.7^{a}	$3.2 \pm 2.6^{b,A}$	$25.7 \pm 8.7^{a,A}$	$0.0 \pm 0.0^{b,A}$	$0.2 \pm 0.2^{b,A}$	14.7 ± 0.4^{A}	17.8 ± 0.4^{A}
Treatment*	309.7 ± 7.5^{B}	344.2 ± 14.9^{B}	39.7 ± 8.5^{a}	$23.7 \pm 10.9^{a,c}$	$45.7 \pm 12^{a,b,B}$	$66.7 \pm 7.8^{b,B}$	$9.5 \pm 1.3^{c,B}$	$21.7 \pm 2.2^{a,c,B}$	26.8 ± 0.5^{B}	31.8 ± 0.5^{B}

a,b,c Numbers with different superscript letters in the same row differ significantly ($P<0.001$)

A,B Numbers with different superscript letters in the same column differ significantly ($P<0.001$)

* Culture medium supplemented with LIF, EGF, GDNF, and bFGF

Table 4 Immunocytochemical staining of goat spermatogonial stem cell using two different markers

Groups	SSC marker (Mean±%)	
	PGP9.5	c-Kit
Control	35.0±1.0[a]	70.6±1.8[a]
Treatment*	71.7±2.8[b]	40.6±1.3[b]

[a,b] Numbers with different superscript letters in the same column differ significantly (*P*<0.001)

* Culture medium supplemented with LIF, EGF, GDNF, and bFGF

In the present study we identified the PGP9.5 positive SSCs in goat spermatogonia during the first wave of spermatogenesis. The pattern of staining was consistent with what has been observed in proliferating pre-spermatogonia in bull and ram testes. The PGP 9.5 positive cells were undifferentiated germ cells located in the basal compartment (Fig. 2a, b) [14, 16].

Considering the effect of culture condition on propagation of SSCs, goat type A spermatogonia were more propagated by addition of growth factors (GDNF, bFGF, EGF, and LIF) compared to the control group (without growth factors). As expected in immunocytochemical staining the proportion of c-kit positive cells was higher in control compared to the treatment group (*P*<0.001). In contrast, the proportion of PGP9.5 positive cells was higher in treatment group compared with control (*P*<0.001). Indeed, the presence of growth factors during culture period could retain the SSCs population in more undifferentiated state (basal SSCs) which was reflected as a higher proportion of PGP9.5 cells in the culture (Table 4).

After 1 and 2 weeks culture period the most type A spermatogonia colonies, grown on the surface of feeder layer, were symmetrical, single, and circle to oval with distinct border. The second group of colonies were chained or rosette form (Fig. 6d-f). It seems the first and second groups of colonies were comprised of basal spermatogonia and differentiating spermatogonia, respectively. They were mostly seen in treatment and control groups, respectively. The addition of growth factors to culture medium increased the number of type A spermatogonia that in turn led to the increment of number and size of colonies. Accordingly, in control group the number and size of colonies were significantly lower than treatment group (Table 3).

In general, the colonies are able to regenerate new colonies upon subculturing [9]. Since in our study condition we just performed the primary culture, we could not see meaningful propagations of colonies at the end of 2 weeks culture period (Table 3). Nonetheless, except for the SSCs colonies <20 μm, there was a positive trend in increment of colony size in both control and treatment groups after 2 weeks culture compared to the 1 week culture period (Table 3).

In conclusion, the present study confirmed the presence of PGP9.5 and c-kit antigens in subpopulation of undifferentiated and differentiated goat type A spermatogonia, respectively. Moreover, the addition of growth factors in

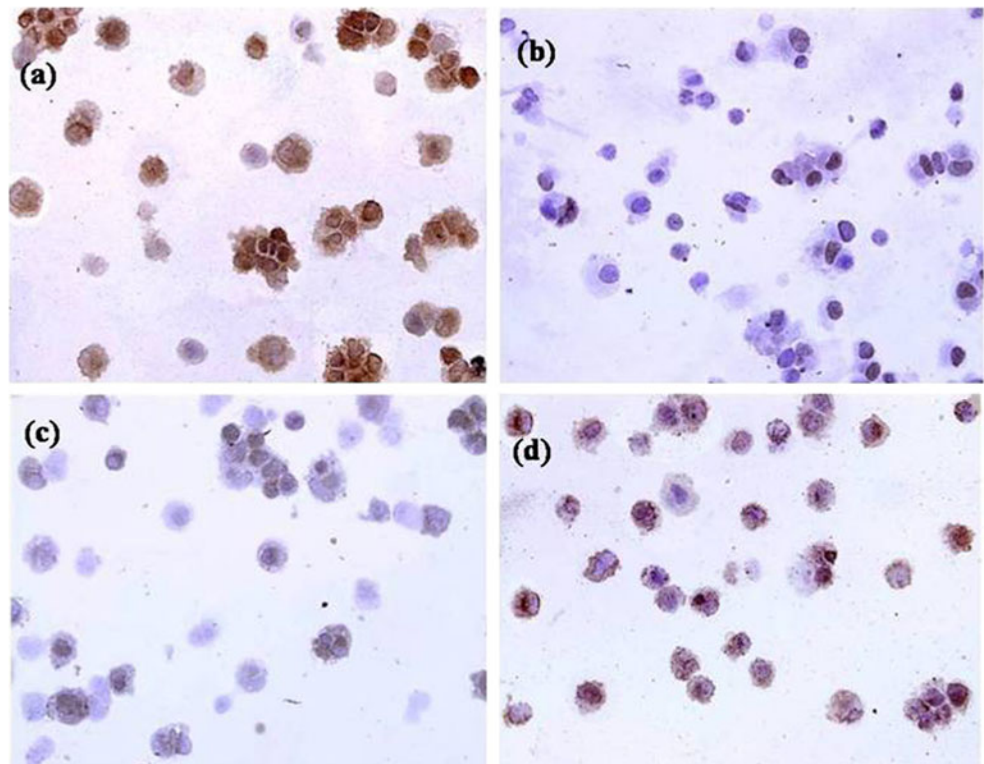

Fig. 7 Immunocytochemical evaluation of PGP9.5 and c-kit positive cells in control and treatment groups. The PGP9.5 positive cells (As, Ap and some Aal), brown cells, in treatment (**a**) group was significantly higher than control (**b**) group. The c-kit positive cells (some Aal, A1-A4), brown cells, in treatment (**c**) group was significantly lower than control (**d**) group

culture medium during short term culture could effectively retain the undifferentiation status of SSCs, reflected as a higher population of PGP9.5 positive cells compared to the c-kit positive ones.

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