PURIFICATION OF SPERMATOGONIAL STEM CELLS FROM RAM TESTICULAR ISOLATE USING FICOLL DENSITY GRADIENT SEPARATION

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ABSTRACT

Spermatogonial stem cells (SSCs) can be used to propagate male superior germ plasm and to preserve the male of high genetic merit and endangered species. The objective was to assess the efficiency of different Ficoll density gradient separation in order to purify SSCs from the testicular cell isolates. The mixed testicular isolates obtained after enzymatic digestion of testicular tissues was subjected to two different Ficoll density gradient methods (method 1: Ficoll at 10 and 12% and method 2: Ficoll at 10, 12 and 16%). The percentage of SSCs (positive for PLZF, an SSC marker) obtained from the fractions, F12 media and F12-16 Interphase (FI) yielded ($p<0.05$) higher percentage of PLZF+ spermatogonia as compared to initial testicular isolate (35.1±3.8 and 22.8±4.5 *vs* 11.2±3.7). The viability (%) of F12 and FI enriched fraction was 55.6±4.3 and 51.2±6.5, respectively. In brief, Ficoll purification method using F12 fraction yielded higher ($p<0.05$) recovery rate ($4.9±1.2x10^6$ cells/g of testis) with improved purity ($1.8±0.4x10^6$ PLZF+ cells) when compared to FI (recovery rate: $3.28±1.2x10^6$ cells/g of testis and purity: $0.8±0.3x10^6$ PLZF+ cells) and can be used either alone or in combination with other purification methods for further enrichment of SSCs that can be used for culture.

Keywords: Ficoll density gradient, PLZF positive, Ram, Spermatogonial stem cell, Testis

INTRODUCTION

Spermatogonial stem cells (SSCs) possess a vital role in male reproduction, genetics and transgenesis studies, however, the successful purification of SSCs from testicular isolate is essential for culture and transplantation (Binsila *et al*., 2017). The testis consists of different types of cells like Sertoli cells, Leydig cells, SSCs and different stages of differentiating germ cells with the percentage of SSCs to be <1% of testicular cells. Attempts were made to isolate and purify SSCs with high viability. Although, the isolation and culture of SSCs has started in bovine, caprine, porcine and bubaline (reviewed in Zheng *et al.,* 2014), the isolation, culture and transplantation of SSCs in livestock is in a nascent stage. Considering the less population of SSCs in the testicular cell isolate, several enrichment procedures were tried to obtain a maximum population of SSCs with appropriate quality which is a prerequisite for downstream applications such as culture experiments and or transplantation. The enrichment procedures include magnetic-activated cell sorting (MACS), fluorescence activated cell sorting (FACS), differential plating, selection with extracellular matrix (ECM), velocity sedimentation and density gradient centrifugation (Borjigin *et al*., 2010; Valli *et al*., 2014). In addition, the combination of enrichment techniques may augment the purity of spermatogonia (Herrid *et al*., 2009). As the initial isolate of testicular cells contains cells of varying size and density, the density gradient separation can be effectively used to enrich SSCs. Recently, the Ficoll density gradient technique provided optimum purity with high viability (Joseph *et al*., 2017). Hence, the objective of present study was to isolate and enrich SSCs from the ram testes using Ficoll density centrifugation.

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MATERIALS AND METHODS

Isolation of putative SSCs: Prepubrtal ram testes samples (n=6) procured from the slaughterhouse were transported within 2 h in ice-cold saline to the laboratory. After removing tunica vaginalis and epididymis, testes tissue samples were excised mechanically, cut into small pieces and testicular cells were isolated immediately using enzymatic methods (Izadyar *et al*., 2002; Borjigin *et al*., 2010) with slight modifications. In brief, testes tissue (2 g) was washed using Dulbecco's Modified Eagles Medium (DMEM) and placed in DMEM. The tissues were mechanically sliced into small pieces, ground with syringe plunger to form homogenized tissue mass. The homogenized samples were added with 4 ml DMEM containing collagenase (2 mg/ml) and incubated for 1 h at 37°C. The digested tissue was washed twice with Dulbecco's phosphate buffered saline (DPBS) by centrifugation at 269 g for 5 min and the supernatant was removed. The pellet containing digested tissues was added with trypsin (0.5 mg/ml) in 5 ml DPBS and incubated at 37˚C for 5-7 min followed by inactivation of trypsin with the addition of an equal volume of DMEM containing 5% fetal bovine serum (FBS). The digested tissues were filtered through 70 µm strainer (352350, BD Falcon) and the filtrate containing cells were washed using DMEM twice by centrifugation at 269 g for 5 min and the supernatant was discarded. The cell pellet containing mixed testicular cells was suspended in DMEM containing 5% fetal bovine serum (Initial cells isolate, IC).

Enrichment using Ficoll density gradient separation: Putative SSCs from the IC were enriched using Ficoll density gradient centrifugation (Panta *et al*., 2011) with modifications. Briefly, two different gradients were carried out viz. Ficoll at 10 and 12% and Ficoll at 10, 12 and 16%. The isolated cells from each gram testes were mixed in 2 ml of 10% Ficoll in DMEM and slowly layered on to the top of 2 ml of 12% Ficoll in DMEM in a 15 ml centrifuge tube. The tubes were centrifuged (5810R, Eppendorf, Germany) at 800 g for 30 min at 18˚C. From 10 and 12% gradient (method 1), 12% Ficoll fraction (F12: pellet and 12% media) and from 10, 12 and 16% gradients (method 2), the interphase of 12 and 16% fraction (FI) were collected. These fractions were enriched in SSCs based on our preliminary study. F12 and FI fractions were centrifuged at 1680 g for 5 min. Supernatants were discarded and the pellet was washed in the media (DMEM added with 5% FBS) by centrifugation at 269 g for 5 min. The supernatant was removed and pellet was re-suspended in the media. Ficoll enriched cells were analyzed for the stem cell property using marker Promyelocytic leukemia zinc finger protein (PLZF), viability and concentration (Figure 1).

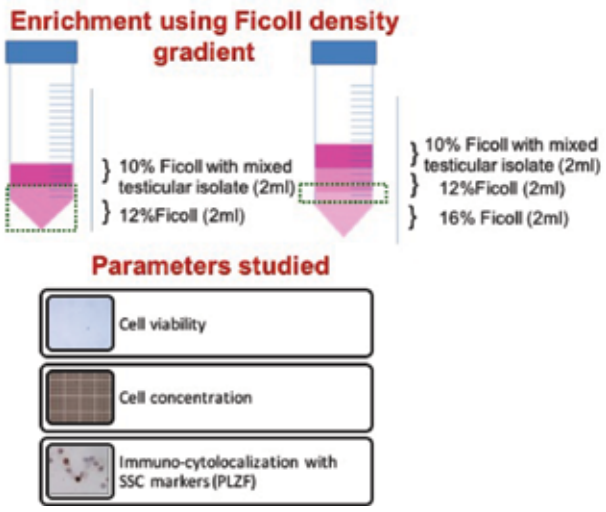

Figure 1: Steps followed for the enrichment of ram SSCs using Ficoll density gradient separation. The initial testicular isolate was subjected to Ficoll density gradient (10 and 12%; 10, 12 and 16%) separation. The SSCs were enriched in 12% Ficoll fraction (F12: Pellet and 12% media; FI: interphase of 12 and 16%).

Localization of different SSC markers: The purity of putative SSCs in different fractions of Ficoll separation was assessed using PLZF (SSC marker) localization in the initial isolate and enriched fractions (Somashekar *et al*., 2017) with slight modifications. Briefly, the smears were fixed with 4% paraformaldehyde for 10 min and rinsed with TBST (Tris-buffered saline with

0.05% Tween 20). Then the slides were incubated in 0.01% Triton-X in TBS for 5 min and washed with TBS. The slides were incubated in TBS containing 0.6% H_2O_2 for 20 min. The nonantigenic sites were blocked by adding a solution of 3% BSA in TBST onto the cells and incubating at room temperature for 30 min. The smear was added with primary antibody (rabbit polyclonal IgG: PLZF, 1:100 dilution, sc-22839, Santa Cruz Biotechnology, Santa Cruz, USA) and incubated overnight at 4°C. After incubation, the slides were washed three times using TBST for 5 min each and incubated with secondary antibody (105499, Goat anti-rabbit IgG-HRP, GeNei, India) for 30 min at room temperature. Then the slides were washed three times with TBST for 5 min each and incubated with DAB substrate for 10 min for the development of brown color. For negative controls, the smears were incubated in the buffer (TBST) without primary antibody. The cells were counterstained with hematoxylin. The cells were observed under 400X magnification using a phase contrast microscope (Nikon 80i, Nikon, Japan) and the percentage of cells positive for PLZF activity was calculated.

Viability of isolated and enriched cells: The viability of the isolated and enriched cells was assessed by trypan blue (0.5%) staining. Equal volume (5 µl) of trypan blue and cells suspension in medium were mixed and incubated for 2 min and observed at 400X magnification in a phase contrast microscope (Nikon 80i, Nikon, Japan). The cells with blue color were considered as dead and those did not take up the stain were considered as viable and the percentages of viability were calculated.

Cell concentration: The number of cells obtained following isolation and enrichments was calculated using hemocytometer and the total number of cells /g of testis were estimated. Based on the immunocytochemistry (ICC) results, the number of SSCs yield /g of testis was calculated in the initial isolate and enriched fraction.

Cell morphology documentation: The cell morphology was estimated in the initial isolate and enriched fractions using a phase contrast microscope (Nikon 80i, Nikon, Japan).

RESULTS AND DISCUSSION

The present study was carried out to enrich ram SSCs from initial testicular isolate using Ficoll density gradient separation. In the method 1, SSCs (PLZF+ cells) were enriched in 12% Ficoll fraction (F12: pellet and 12% media; 35.1±3.8%) and using the method 2, Ficoll at 10, 12 and 16% gradients, the SSCs were enriched in the interphase of 12 and 16% fraction (FInterphase; 22.8±4.5%; Table1). The percentage of PLZF+ cells in F12 fraction was higher ($p<0.05$) than the initial isolate, however, did not differ ($p>0.05$) with FI fractions. The morphologically enriched cells were round in shape with a homogenous size (Figure 2a). In the present study, 3.6 (FI) and 4.1 (F12) Fold enrichment obtained through Ficoll gradient separation

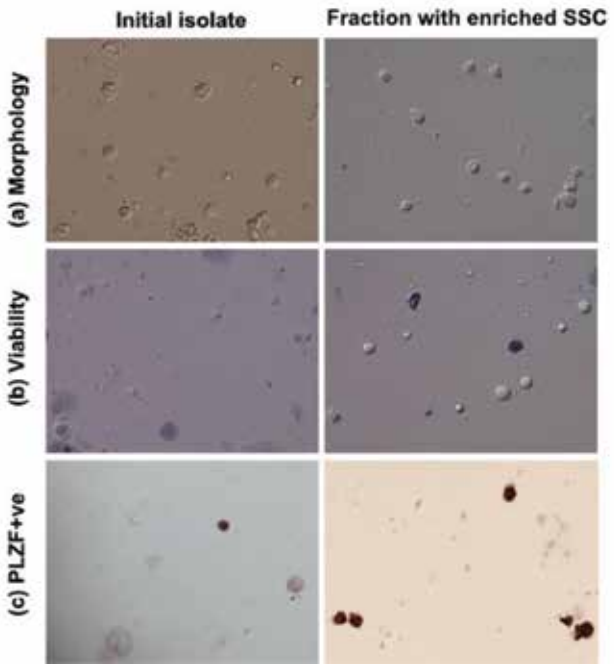

Figure 2: (a) Morphological appearance of pre-enriched and enriched (round in shape with a homogenous size) SSC fractions, (b) The cells stained with trypan blue are dead and others are live, and (c) The putative SSCs (brown colour) were identified using PLZF marker. 400x

Table 1: Enrichment of putative spermatogonial stem cells (SSCs) from the initial ram testes isolates (n=6) using Ficoll density gradient centrifugation. Method 1: 12% Ficoll fraction (F12: pellet and 12% media), Method 2: Interphase of 12% and 16% fraction (FI). PLZF, Promyelocytic leukemia zinc finger protein.

Parameters	Initial isolate	Ficoll enrichment	
		Method 1	Method 2
Total no. of cells (x10^6) /g of testis	17.7±2.8	4.9±1.2	3.3±1.2
Viability, %	72.0±4.1^{c}	51.2±6.5^{ab}	55.6±4.3^{a}
PLZF^{+ve}, %	11.2±3.7^{a}	35.1±3.8^{bc}	22.8±4.5^{ab}

^{a,b,c}p<0.05

is in agreement with the 2.9 fold in bovine (Herrid *et al*., 2009) and 3.6 fold in sheep (Rodriguez-Sosa *et al*., 2006) through percoll enrichment. Further, the inclusion of density gradient separation along with differential plating method improved the purity of SSCs (42.70 to 64.39% GFRα+ cells) in cats (Tiptanavattana *et al*., 2015).

The viability of purified fraction following Ficoll density gradient though reduced (p<0.05) as compared to initial isolate, did not adversely affect the culture procedure (unpublished data; Table 1, Figure 2b). Similarly, an earlier study reported reduced viability of enriched bovine SSCs obtained through Percoll density gradient centrifugation (de Barros *et al*., 2012). The average yield of number of mixed testicular cells from each gram of testicular tissue was 17.67x10^6 and

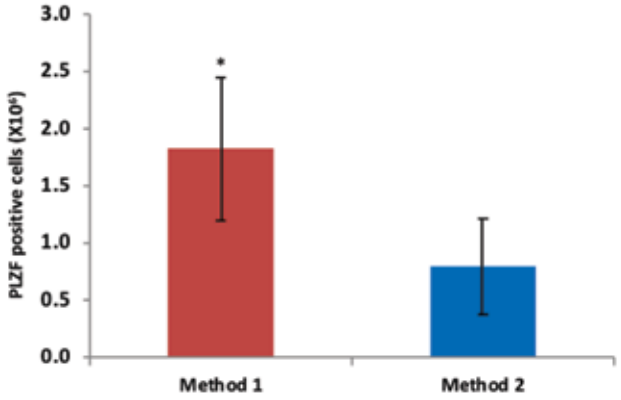

Figure 3: Total number of PLZF^{+} cells (x10^6) in 12% Ficoll fraction (F12: pellet and 12% media, Method 1) and in the interphase of 12% and 16% fraction (FI, Method 2) of ram testes isolates (n=6). *p<0.05.

the purified fractions, F12 and FI yielded 4.85x10^6 and 3.28x10^6 cells from prepubertal ram, respectively (Table 1). The total number of PLZF+ cells in F12 and FI fractions were 1.8±0.4 and 0.8±0.3 million, respectively (Figure 3).

The study revealed that even though there was no significant difference in PLZF+ cells between the two enriched fractions (F12 and FI), the F12 fraction was better based on higher recovery rate. Hence, F12 fraction of 10 and 12% Ficoll density gradient may be used for the culture of SSCs. Through PLZF localization study, it was revealed that SSCs can be enriched by Ficoll density gradient separation and the procedure is suitable for eliminating the other testicular cells like differentiating spermatogonial population and Sertoli cells as these cells will be separated out in different layers based on the density (Figure 2c). A similar study for the enrichment of SSCs using Ficoll density gradient was reported in rodent (Jeong *et al*., 2003) and fish (Panda *et al*., 2011). Percoll density gradient though chemically different, but separates cells based on density and has been used for SSCs isolation in different studies (Izadyar *et al*., 2002; Tiptanavattana *et al.,* 2015). As the initial isolate of the testicular cells contains cells of varying size and density, density gradient separation might be a suitable option to segregate the subsets of homogenous cells. The present study, though provide the optimum number of PLZF positive cells for culture, the SSCs enriched fraction from density gradient separation can be combined with other protocols like differential plating (Izadyar *et al*., 2002; Tiptanavattana *et al*., 2015) or

magnetic activated cell sorting (MACS) (Panda *et al*., 2011) for improving enrichment efficiency. Another concern from the experiment is the reduced viability of isolated cells. The viability of the cells may be probably improved by reducing the duration of experimental procedures.

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