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Research Article

Isolation and Enrichment of Spermatogonial Stem Cells with MACS in Cattle Calves by Autopsy in Vitro

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ABSTRACT

Spermatognial stem cells (SSCs) are unipotent adult stem cells which form the foundation of spermatogenesis for continuous production of sperms throughout the animal life. Due to their rare availability in the testis, we decided to target CD9 surface marker and magnetic–activated cells sorting MACS for enrichment of testicular cell suspension with the cells of stem cell properties. We have successfully characterized the isolated and enriched SSCs population with the Fluorescein isothiocyanate FITC tagged secondary antibody under fluorescent microscope at 500 nm wavelength. Isolated Cells could form the colonies resembling to SSCs colonies after one week of culturing in DMEM along with 10% ABS at 37^0C temperature, 5% CO_2 and 90%NO_2. α–*N*–acetylgalactosamine (GalNAc) is reported to be present in SSCs. We have used FITC– DBA lectin to characterize the SSCs colonies after 14 days of culture. Our findings suggest the use of CD9 surface marker and MACS technology for the enrichment of SSCs and DBA for characterizing SSC colonies.

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INTRODUCTION

Stem cells have the potential to revolutionize tissue regeneration and engineering. The hematopoietic stem cells were the first stem cells to be prospectively identified. Since then, an ever increasing number of new types of stem cells, including embryonic stem cells, spermatogonial stem cells (SSCs) and cancer stem cells, have been identified and characterized. They can be useful in understanding general cellular processes in, e.g., embryogenesis, organogenesis, cancer or ageing, but also as a vehicle for the generation of transgenic animals for functional gene analysis and disease models (Bosio et al., 2009).

Spermatogonial stem cells (SSCs) are unipotent adult stem cells responsible for the maintenance of the spermatogenesis throughout the entire life of the male (Bosio et al., 2009). They are the only germ line stem cells in adult animals. The SSC may choose to self–renewal or generate a daughter cell committed to differentiation (Van der and Weiss, 2000). They form the foundation of spermatogenesis and are required for the continuous production of sperm through a balance between SSC self–renewal and differentiation in adult testis (Hofmann, 2008). Studies of SSCs are complicated because these cells are very few in number (Aponte et al., 2005). This provides an opportunity for methods like magnetic–activated cells sorting (MACS) and fluorescence activated cells sorting (FACS) which can enrich whole cell suspension with SSCs. SSCs provide the foundation for spermatogenesis throughout the life of male animals (De Rooij and Russell, 2000).

Kanatsu–Shinohara et al. (2003) reported the isolation and long–term culture system for SSCs. Development of this culture systems provided possibilities to study SSCs *in vitro*. However, the percentage of SSCs in GS cell culture was unexpectedly low, and only 0.04–1.26% could colonize and reconstitute seminiferous tubules of infertile animals (Kanatsu–Shinohara et al., 2005).

For isolation of spermatogonial stem cells (SSCs) and undifferentiated spermatogonia, Dym et al. (2009) studied certain markers including CD9 and OCT–4 in mammals. He et al. (2010) isolated human spermatogonial cells by MACS, using GPR125 as a specific marker for spermatogonial cells in mouse. Recently Piravar et al. (2013) have reported the efficient use of MACS using various surface markers such as CD 9, OCT–4 etc. to enrich and culture the SSCs *in vitro* without feeder cell lines. FACS and MACS are the techniques which had been used in past to enrich the SSCs population. Zhao et al. (2002) and De Wynter et al. (1995) also have used MACS to purify and enrich of stem cells targeting various surface markers and found remarkable success in their experiment.

Lectins proteins such as peanut agglutinin (PNA), which recognizes D–(+)– galactose, has been used to fractionate bovine hematopoietic stem cells and also in the purification and identification of adult neural stem cells (Salner et al., 1982). In this experiment, we describe Dolichos biflorus agglutinin (DBA), which recognizes α–*N*–acetylgalactosamine (GalNAc) as being highly reactive towards bovine SSCs. More recently Nash et al. (2007) reported the utility for DBA in the characterization of

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pluripotent cells because it can be used as a nondestructive marker and as a reliable readout for initial differentiation events, at a level of temporal resolution that was not previously possible.

A number of different protocols have been published for the isolation and enrichment of stem cells including selective culturing, immunopanning, flow cytometric sorting, or magnetic sorting. Based on the available literature, we hypothesis that CD9 can serve as a good surface marker for enrichment by MACS. In this article, we discussed not only about the isolation procedures of SSCs but also about their enrichment with the help of magnetic assisted cell sorting because they are present only in small number in the testes. The colonies formed from SSCs were characterized by FITC tagged DBA lectin protein.

MATERIAL AND METHODS

Collection of Testes

The testes were collected from the recently dead cattle calves (6–12 months old) by autopsy. The testes were brought to laboratory in the phosphate buffer saline supplemented with penicillin and streptomycin, under cold conditions within 2hrs.

Isolation of Spermatogonial Stem Cells (SSCs)

Visible connective tissue was first removed from the testis and then testes were washed three times in 0.9 % normal saline containing antibiotics and tunica albuginea was removed. About 10–15 g of testicular sample was processed for SSCs isolation. The chopped testicular samples were processed for enzymatic digestion as described by Van Pelt et al. (1996) with minor modifications. In brief, the testicular sample was chopped and minced into tiny pieces and later suspended in DPBS solution for washing. At last, the minced sample was suspended and washed with DMEM containing 14 mol NaHCO3/L, 4 mol Lglutamine/L, 1 ml/100 ml single–strength non–essential amino acids, 1000 IU/ml–100 µg/ml penicillin–streptomycin and 15 mol/L Hepes. The Sample was then suspended in digestion media [DMEM containing 1 mg/ml collagenase and 5 µg/ml DNase I (all from Sigma– Aldrich, St. Louis, MO, USA)] and incubated at 37°C for 45 min in a shaking water bath operated at 140 cycles/min at 37^0C. After three washing in same medium the most of the interstitial cells were removed in supernatant. Seminiferous cord fragments were then given second digestion with 1 mg/ml collagenase, 1 mg/ ml hyaluronidase and 5 µg/ml DNase for 30 min. The sample was processed for third digestion with collagenase (0.5mg/ml), hyaluronidase (0.5mg/ml) and DNase (2.5 µg/ml) for thirty minutes. All the digestion steps were performed in water bath @120–140 cycles/minute at 37^0C. The dispersed cells were transferred into a beaker kept in ice and then washed twice in DMEM medium supplemented with 10 % (v/v) adult bovine serum (ABS) to stop the enzymatic digestion. The cells were separated from the remaining tubule fragments by centrifugation at 500g for 5 min and after filtration through 100 and 60 µm nylon filters (Millipore Corp., Bedford, MA, USA), the cells were pelleted. The isolated cells contained mixed population of cells of both the somatic cell population and stem cells.

Enrichment of Spermatogonial Stem Cells (SSCs) with MACS

After enzymatic digestion, one part of the suspended cells at the concentration of 1–2 x 10^6 cells/ml was seeded in each well of culture plates. DMEM along with ABS (10%) was used as culture medium.

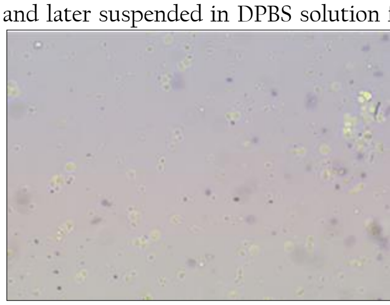


Figure 1a

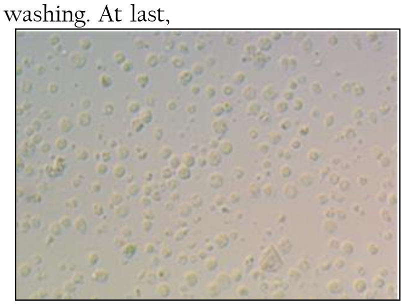
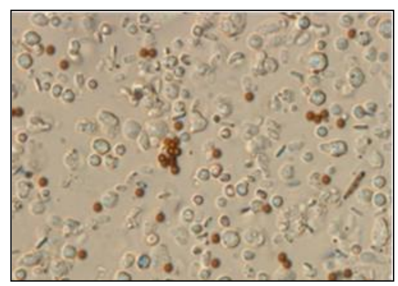


Figure 1b



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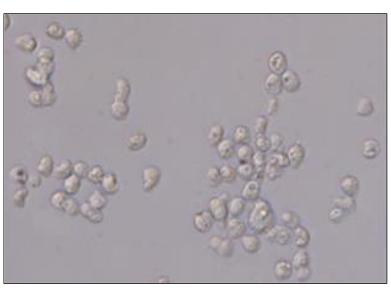

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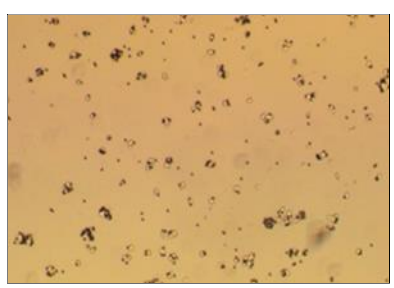

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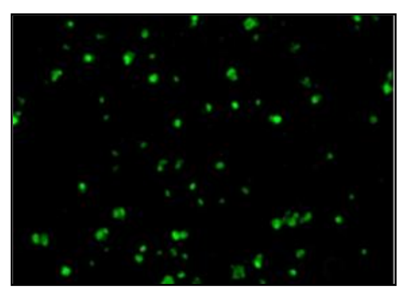

Figure 1f

Figure 1a: 100 X live (color less) and dead (blue) in single cells suspension; Figure 1b: 100 X 0 day cultured cells where the isolated viable testicular cells are seeded; Figure 1c: 100 X microbeads attached with CD9+ SSC; Figure 1d: 100 X microbeads detached from CD9+SSCs; Figure 1e: 100 X SSCs bound with microbeads attached with FITC Tagged Secondary antibody under light microscopy; Figure 1f: 100 X SSCs bound with microbeads attached with FITC Tagged Secondary antibody (green) under fluorescent Microscope

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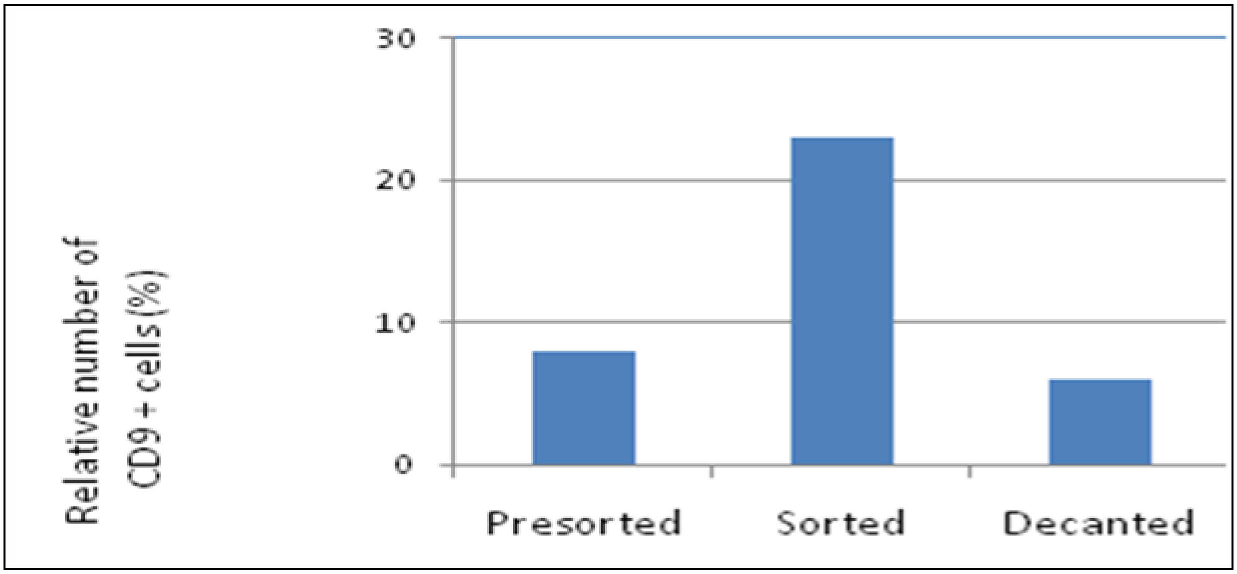

Figure 2a

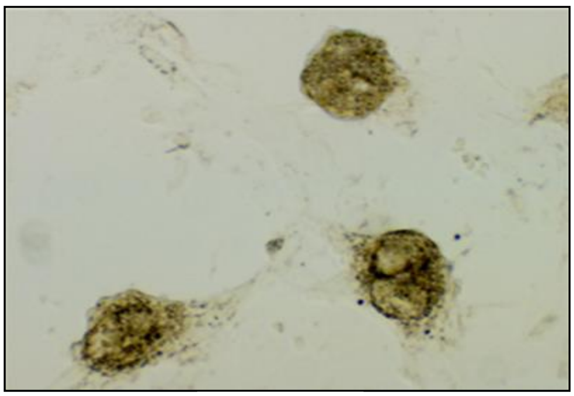

Figure 2b

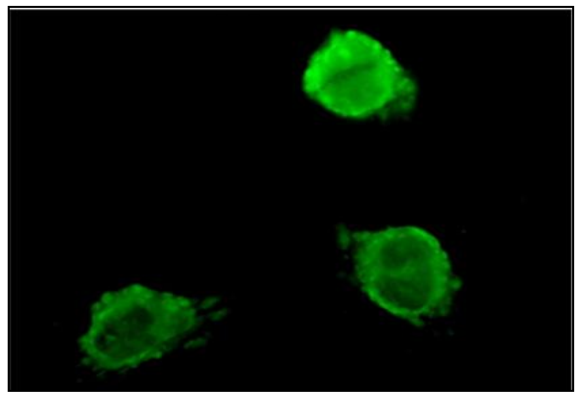

Figure 2c

Figure 2a: Relative difference in number of unsorted and sorted SSCs; Figure 2b and 2c: 100 X immunostaining of 14 days old SSCs colonies probed with FITC tagged DBA lectine protein under light (figure 2b) and florescent (figure 2c) microscope

The culture conditions were kept as 37^{0}C temperature, 5% CO_2 and 90%N_2. While another part of the suspended cells were processed for magnate assisted cell sorting (MACS) as it was described by Bosio et al. (2009) with slight modifications. CD–9 cell surface marker was targeted to enrich the cell suspensions with stem cells. The CD9+ SSCs were obtained using the MACS kit according to the manufacturer's instructions (Invitogen). The cells were incubated with 100 µl microbeads directly conjugated to mouse monoclonal anti–human CD 9 antibody at 4°C for 30 min. Subsequently, the suspended cells were added to a MACS column that was placed in the magnetic field of a MACS separator (Invitrogen). The labeled CD9 positive cells were retained on the column and the unlabeled cells were eluted; when the column was removed from the magnetic field, the magnetically retained CD9+ cells were collected as positively selected cells for further research (He et al., 2011). Microbeads were detached from purified cells by incubating in Dulbecco modified minimum essential medium (DMEM) + 5% fetal calf serum (FCS) for 6hrs at 37^{0}C. These cells were washed with DMEM + 10% ABS (adult bovine serum) three times.

Phenotypic Characterization of SSCs Sorted by MACS

CD–9 cell surface marker was targeted to enrich the cell suspensions with stem cells. Primary antibodies (0.5µg) against CD–9 (Invitrogen, AHS0902) surface markers were added to the cell suspension (1 x 10^6/ml) and incubated for 10–15 minutes at 2–8^{0}C. After the incubation, the cells were washed three times with buffer–1 (PBS+1% BSA, pH–7.4) and 1 x 10^6 (25µl) microbeads bound with FITC tagged secondary antibody (Invitrogen, 110.31) were added. The cell suspension along with buffer and microbeads attached with FITC tagged secondary antibody was incubated for 30 min with continuous rotation @ 10 rotation per min at 2–8^{0}C.

Phenotypic Characterization of 14 Days Old Colonies with FITC Tagged DBA Lectin

Fluorescent–labeled lectins were purchased from Invitrogen. Colonies formed after 14 days of culturing SSCs were stained with Fluorescein isothiocyanate (FITC) tagged lectin DBA with slight modifications from methods used by Nash et al. (2007). SSCs colonies were fixed with 4 % para formaldehyde for 30 minutes at room temperature after five times washing with Dulbeccos modified phosphate buffer saline (DPBS). Washing with DPBS (five minutes each) was repeated three times after fixing the colonies. The SSCs colonies were incubated in triton–x–100 (0.1%) for 30 minutes at room temperature. Colonies were washed again three times with DPBS. Non specific sites were blocked with goat serum (4% in DPBS) at room temperature for 30 minutes. Blocking solution was decanted and FITC–DBA was added to the cultured colonies for 1–1.5 hr at room temperature in dark. Just before observation under fluorescent microscope at 500nm the colonies were washed three times with DPBS.

RESULTS

Isolation of SSCs

Cell viability (Figure 1a) was found to be more that 80% in the single cell suspension after enzymatic digestion while the whole cell concentration was found to be 1–2 x 10^7/ml Single cell suspension was obtained without any undesired material from testis and seeded in each well at the concentration of 1–2 x 10^6/ml (Figure 1b).

Enrichment of SSCs with MACS

The micro beads could attach to CD9+ SSCs (Figure 1c). CD9+ SSCs were increased apparently in number after being sorted with MACS (Figure 2a).

Detachment of Microbeads from SSCs

The microbeads which were bound with CD9+ SSCs were removed completely for further culture (Figure 1d).

Phenotypic Characterization of SSCs with FITC Dye

The cells which were bound with microbeads were further processed for phenotypic characterization using FITC tagged secondary antibodies. They were photographed under light microscope and florescent microscope and green color florescent was obtained from the same cells where the microbeads were bound (Figure 1e and Figure 1f respectively).

SSCs Colonies and their Characterization with DBA

The MACS sorted CD9+ SSCs formed colonies which were characterized further by FITC tagged DBA lactin protein (Figure 2b Figure 2c).

DISCUSSION

The number of stem cells in testes is very low. According to Aponte et al. (2005), SSC comprise only 0.03% of all germ cells in testis and their isolation is often hindered by the presence of spermatogonial cells at different stages of differentiation. Our previous experiments conducted on the different age group of animals revealed that as the age of the animal increases the number of SSCs in testis decreases (unpublished data). Shinohara et al. (2000) have used CD9 cell surface marker to purify the SSCs form mixed cell population obtained from testis. Viability of the cells is another important criterion for culturing the SSCs. Viability of the cells decreases as the time lapses after animal's death due to autolysis of the cells. Van Pelt et al. (1996) also mentioned about the importance of time gap between the death and isolation of SSCs. The testes were therefore, collected within 2 hr after the death of the animal. More than 80 % viability and 1–2 x 10^6Cells/ml concentration resulted in a very few (2–3) colonies resembling to SSCs colonies. The SSC isolated from seminiferous tubules is often contaminated by differentiating spermatogonial cells, sertoli cells and peritubular myoid cells. Most SSC culture systems are known to contain a mixture of testicular cells with about 1.33% SSC (Aponte et al., 2005). Luo et al. (2006) have used MACS to isolate the SSCs mixed cell population because identification and isolation of SSC had been difficult due to their rarity in testis and lack of SSC specific cell surface markers. Rodriguez–Sosa et al., (2006) also employed various methods such as differential plating, velocity sedimentation, elutriation, discontinuous gradient, Hoechst 33342 and rhodamine 123 side population, magnetic–activated cells sorting (MACS) and fluorescence activated cells sorting (FACS) to isolate SSC in different species. We could observe the direct effect of pH of the buffer used in attachment and detachment of microbeads with the cells. Buffer with pH 7.4 gave good binding results in comparison to the buffer with pH 7.2 and pH7.6. Li et al. (2013) reported that even after enriching the SSCs with percoll density gradient method, the cell suspension was still containing few contaminating testicular cells. Hence we used CD9 surface marker along with MACS to enrich the cell population with the SSCs. Similar to Kala et al. (2012), we also used FITC attached with secondary antibodies to characterize isolated SSCs under florescent microscope to confirm the purity of isolated CD9 positive SSCs.

Lengner et al. (2007) observed very low levels of OCT–4 expression in SSCs as compared with ESCs. Hence, it cannot serve as a good marker for enrichment of SSCs. Kanatsu–Shinohara et al. (2004) concluded that CD–9 is a better surface marker in comparison to OCT–4 in testicular samples. We found that MACS can be used to enrich the culture with stem cell properties if the surface markers are known. Recently, Sheng et al. (2013) have also used MACS to enrich prostate cancerous cells which have the stem cell like properties. Patrawala et al. (2006) and Guo et al. (2012) have also used MACS for enrichment of stem cells in combination with antibodies against CD 9 surface marker. CD 9 is a stem cell surface marker which may act as a good choice of surface marker to enrich stem cells along with MACS (Cui et al., 2004). We were able to get relatively good number of CD9 positive cells when the cell suspension was sorted with MACS.

DBA also has been used as a marker for prespermatogonia, the precursors of bovine spermatogonia present until the onset of spermatogenesis at week 30 of age (Ertl et al., 1992). Here, we have hypothesized that DBA will be expressed in the SSCs of young bulls aged around 6 months. Hence, the colony formed from the SSCs isolated from young calves should be positive for presence of DBA marker. Our results were in support of our hypothesis where colonies were found to be positive for FITC–DBA. Nash et al. (2007) also reported the stem cells exhibiting affinity towards lectin DBA protein.

CONCLUSION

MACS in combination with stem cell marker CD9 provide an efficient tool for enrichment of cattle calves' SSCs. Further research may be carried out to compare the efficacy of FACS and MACS.

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