In vitro culture and morphological characterization of prepubertal buffalo (*Bubalus bubalis*) putative spermatogonial stem cell

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Abstract

Purpose Spermatogonial stem cells (SSCs) have the unique ability both to self-renew and to produce progeny that undergo differentiation to spermatozoa. The present study has been carried out to develop a method to purify and enrich the pure populations of spermatogonial stem cell like cells in buffalo.

Methods The spermatogonial cells were isolated from testes of 3–7 month old buffalo calves and disaggregated by double enzymatic digestion. Mixed population of isolated cells were then plated on *Datura stramonium* agglutinin (DSA) lectin coated dishes for attachment of Sertoli cells. The desired cells were obtained from suspension medium after 18 h of incubation and then loaded on discontinuous density gradient using percoll (20–65 %) and different types of spermatogonia cells were obtained at interface of each layer. These cells were cultured in vitro.

Results Spermatogonial cells isolated have spherical outline and two or three eccentrically placed nucleoli, created a colony after proliferation during first week or immediately after passage. After 7–10 days of culture, the resulted developed colonies of spermatogonial cells expressed the spermatogonial specific genes like Plzf and VASA; and other pluripotency related markers viz. alkaline phosphatase, DBA, CD9, CD90, SSEA-1, OCT-4, NANOG and REX-1.

Capsule Present study showed the in vitro culture of enriched buffalo spermatogonial stem cell like cells and thereby characterized for some of the pluripotency markers.

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Conclusion Our results show that the isolated putative spermatogonial stem cells exhibit the expression of pluripotency related and spermatogonial specific genes. This study may help to establish a long term culture system for buffalo spermatogonia.

Keywords Spermatogonia stem cells · Pluripotency · Self renewal · Testis · Sertoli cells

Introduction

Spermatogenesis is a dynamic process in which stem spermatogonia through a series of events involving mitosis, meiosis and cellular differentiation become mature spermatozoa. Sertoli cells are in close contact with germ cells in the seminiferous tubule; they play a critical role in these processes by providing the germ cells with physical support, nutrients, hormonal and paracrine signals, all of which are necessary for successful spermatogenesis [9]. At birth the seminiferous tubules only contain Sertoli cells and fetal germ cells, called gonocytes. At some point of postnatal development some gonocytes directly form A1 spermatogonia (a time point called the start of spermatogenesis), which proliferate and develop into progressively more differentiated cell types, produce spermatocytes that carry out meiosis, give rise to spermatids and eventually formed sperm [26]. At puberty, the cycle of the seminiferous epithelium becomes normally established and continues steadily, increasing its efficiency as the animal becomes sexually mature. However, recent reports suggest that these cells have a greater potential. Testes of both neonatal and 6-week-old mice were reported to contain

pluripotent cells or cells able to express pluripotent properties in vitro [10, 14, 28].

These cells have been shown to be harvested successfully from a testis and introduced into another testis where they resume spermatogenesis [4]. They can also be cryopreserved for later use [3]. Over the years, culture conditions for mouse spermatogonial stem cells have been established, facilitating the characterization of these cells and factors involved in self-renewal and differentiation [21]. In spite of their biological significance, the behavioural and molecular mechanisms underlying the behaviour of undifferentiated type A spermatogonia have not been elucidated largely because of the lack of a specific biological marker. POU5F1 (previously known as Oct4), a homeobox transcription factor that is required for the maintenance of pluripotency of embryonic stem cells [19, 20], is expressed in undifferentiated spermatogonia and can be called as SSCs [24]. A Sry-related HMG box transcription factor, Sox3, was found to be expressed specifically in undifferentiated type A spermatogonia [25, 32]. Another nuclear protein, promyelocytic leukemia zinc-finger (ZBTB16; previously known as Plzf), which functions as a transcriptional repressor, also was reported to be expressed only in undifferentiated type A spermatogonia [5, 6]. These molecules are candidate markers for undifferentiated type A spermatogonia; however, the cells expressing these markers cannot be separated alive by immunologic methods because of the markers' nuclear localization. Studies have validated that *Dolichos biflorus* agglutinin (DBA) stains a population of cells in the bovine testis with morphological and positional characteristics of spermatogonial stem cell [22].

Defining the factors that regulate the balance of self-renewal and differentiation of SSCs is of great interest; we sought to determine whether SSCs cells would provide a suitable system for investigating this matter using morphological evaluation. In the present study, we have succeeded in deriving SSCs from prepubertal buffalo calf and long term in vitro culture of SSCs. Morphological examination of SSC cells confirmed their resemblance to spermatogonia of the undifferentiated type. The crucial first step in achieving these goals is by establishing a method for isolating a cell fraction enriched with undifferentiated spermatogonia from bull testis and its in vitro proliferation and its survival.

Material and methods

Collection of testis samples

Testes from 3 to 7 month-old buffalo calves were obtained from local abattoir, and transported to the laboratory in normal saline supplemented with penicillin and streptomycin, under cold conditions within 2 h, for isolation of spermatogenic

cells. Visible connective tissue was first removed from the testis and then washed three times in 0.9 % normal saline containing antibiotics and removed tunica albuginea.

Isolation of spermatogonial cells

Isolation of spermatogonial cells was carried out as described by van Pelt et al. [31] with minor modifications. Testes were decapsulated and 3–5 g of tissue was collected and minced into small pieces suspended in Dulbecco's modified Eagle's medium supplemented with 14 mol NaHCO₃/L, 4 mol L-glutamine/L, 1 ml/100 ml single-strength non-essential amino acids, 100 IU/ml–100 µg/ml penicillin–streptomycin, 40 µg/ml gentamycin and 15 mol/L Hepes. Minced tissue were then suspended in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase type II, 5 µg/ml DNase I and 1 mg/ml trypsin (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. 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 collagenase, hyaluronidase and DNase with same concentration for 30 min as described above. The dispersed cells were then washed twice in medium supplemented with 10 % (v/v) fetal bovine serum (FBS) to stop the enzymatic digestion. Cells were separated from the remaining tubule fragments by centrifugation at 1,000 rpm for 5 min and after filtration through 80 and 60 µm nylon filters (Millipore Corp., Bedford, MA, USA), the cells were pelleted. The isolated cells contain mixed population of cells containing both the somatic cell population and stem cells. Spermatogonial stem cells were separated from this mixed cell population by differential plating and Percoll density gradient.

Differential plating

Differential plating was used to separate sertoli cells from isolated cell population as described by Scarpino et al. [27]. Petri dishes with a diameter of 35 mm were coated with a solution of 5 µg/ml of *Datura stramonium* agglutinin (DSA lectin) from Sigma-Aldrich in Dulbecco's phosphate-buffered saline (DPBS) at 37 °C for 1 h. Coated flasks were then gently rinsed three times with DPBS; DPBS +0.5 mg/ml bovine serum albumin (BSA) was added and incubated for 1 h at 37 °C to reduce non-specific binding of cells. Petri dishes were again washed three times with DPBS and kept moist with PBS until required. Prior to the addition of isolated cells, PBS was replaced by DMEM +10 % FBS. The mixed population of the cells obtained by enzymatic digestion was placed on lectin-coated dishes and incubated for overnight at 37 °C in a humidified atmosphere of 5 % CO₂ in air with 20×10⁶ cells per dish. After incubation, the non-adhering cells so obtained

were subjected to percoll density gradient separation for further enrichment. The adhering cells were allowed to grow till 70–80 % confluency. Sertoli cell feeder monolayer was made mitotically inactive by treating with mitomycin C.

Cell separation with discontinuous density gradient

An iso-osmotic Percoll (Sigma-Aldrich) suspension was prepared containing 82.2 % Percoll in DMEM, 0.6 % BSA and 45 µg/ml DNase. A discontinuous density gradient was made by diluting the iso-osmotic Percoll suspension with DMEM, 0.7 % BSA and 50 µg/ml DNase. The percentages of Percoll with respective densities were 65 %, 50 %, 40 %, 36 %, 34 %, 32 %, 30 %, 28 % and 20 %. The gradient was built up in a polypropylene tube (round bottom; 100 mm long; outer diam. 17 mm, inner diam. 14 mm) with 1 ml of each Percoll density suspension. The cell suspension was layered on top of the gradient in 500 µl DMEM, 0.7 % BSA, and 50 µg/ml DNase. The gradient was centrifuged at $800 \times g$ for 30 min at 18 °C. Cells found in the interface between the different- density suspensions were collected as fractions 1–10. Cell viability was measured at each step of purification.

Spermatogonial cells cultured with growth factor and co-cultured with sertoli cells

SSC-enriched cells so obtained after percoll density gradient centrifugation were cultured on sertoli cell feeder layer at 37°C in a humidified atmosphere of 5 % CO₂ in air. Culture medium consisted of HEPES modified DMEM supplemented with 10 % (v/v) FBS, 14 mol NaHCO₃/L, 4 mol L-glutamine/L, 1 ml/100 ml single-strength non-essential amino acids, 100 IU/ml–100 µg/ml penicillin–streptomycin, 40 µg/ml gentamycin and 15 mol/L Hepes. Culture medium was changed twice per week.

The cells were grown with different concentrations of GDNF for 4 week. The SSC culture groups include (1) control group, (2) SSCs co-cultured with Sertoli cells and (3) GDNF supplementation (consisted of three groups, received 10, 50, and 100 ng/ml of fresh GDNF (Invitrogen Tech-line, West Bend, WI), respectively, every 3 day for 4 week). The diameters and the number of colonies were determined every 7 day during the culture for 4 week. An inverted phase contrast microscope (Nikon, Microphot, FXA, Japan) was used to determine the number of the colonies.

Alkaline phosphatase activity and immunofluorescence staining

For alkaline phosphatase staining, the medium was removed and SSC colonies were given DPBS washing twice. The cells were fixed in citrate-acetone-formaldehyde fixative

solution for 1 min. After fixation, the cells were washed 3 times with deionised water for 1 min and incubated for 15 min at room temperature with the alkaline dye. The cells were rinsed again 2–3 times with deionised water and counter stained with neutral red stain for 1–2 min. Finally, colonies were washed several times to remove the extra neutral red stain and the response of the cells to alkaline phosphatase staining was observed under a microscope.

The expression of intracellular markers likes surface markers; DBA-FITC labelled, OCT 4, SSEA-1, CD9 for SSC colonies and vimentin for sertoli cells was examined by immunofluorescence staining. The colonies were fixed in 4 % paraformaldehyde in DPBS for 30 min, washed 3 times with DPBS and then permeabilized by treatment with 0.1 % Triton X-100 in DPBS for 30 min. After thoroughly washing with DPBS, the cells were incubated with the blocking solution (4 % normal goat serum) for 30 min, and then with the primary antibody at a dilution of 1:10 to 1:20 for 1 h. In the respective negative controls, the addition of the primary antibody was omitted. After washing 3 times with DPBS, cells were incubated with the appropriate FITC-labeled secondary antibody (anti-mouse IgG and anti mouse IgM, diluted 1:50 to 1:100) for 2 h. The cells colonies were then examined under a fluorescence microscope (Nikon, Tokyo, Japan).

Expression of the transcription factors in cells

RNA isolation and cDNA amplification

The total RNA was isolated from cultured cells using the “Cells-to-cDNA kit-II” (Ambion, Austin, TX, USA) as per manufacturer’s instructions. Briefly, the clumps of cells were taken in tubes after washing with 1x ice cold PBS. Washed cells were transferred to 100 µL of cold lysis buffer and incubated in a thermal cycler at 75 °C for 10 min. The cell lysate was treated with DNase-I at 37 °C for 30 min to degrade genomic DNA and then heated at 75 °C for 5 min to inactivate the DNase-I. The cell lysate (10 µL) was used for making cDNA using random decamer primer to analyze expression of the genes OCT4, SOX-2, NANOG, REX- 1, PLZF and VASA with primers as shown (supporting information in Table 1). GAPDH were used as internal control. A negative RT reaction (parallel RT reaction but without MMLV enzyme) was set with every batch of cDNA preparation to check genomic DNA contamination and a negative PCR without template was also set.

Spectral karyotyping

Chromosomal integrity of SSCs -like cells were analyzed by performing karyotyping. The SSCs-like cells were incubated with 0.1 µg/ml colcemid for 4 h at 37 °C. The colonies were

Table 1 Primers used for gene expression studies

Gene	Primer sequence (5'- 3')	Annealing Temp/Cycle	Product size (bp)	Gene Acc. No.
OCT-4	5'-GTTCTCTTTGGAAAGGTGTTTC-3' 5'-ACACTCGGACACGTCTTTC-3'	57°C/35	341	AF022987
VASA	5'-GGAAGTGGACGAGGTGGTTA-3' 5'-GTCTGAGCACAAAGCCATCAA-3'	53°C/35	389	AF541971.1
NANOG	5'-GGGAAGGGTAATGAGTCCAA-3' 5'-AGCCTCCCTATCCCAGAAAA-3'	57°C/35	211	DQ487022
PLZF	5'-ATGGACTTCAGCACCTACGG-3' 5'-CTTCCCACACAGCAGACAGA-3'	53°C/35	396	BT026530.1
REX-1	5'-CAGCTGCCATAGGCTCTACC-3' 5'-GTCTGGCCTGACAAAGGTGT-3'	55°C/35	239	XM_001255545
GAPDH	5'-ATCACCCCTGCTTCTACTGG-3' 5'-AGGAGACAACCTGGTCCTCA-3'	60°C/35	235	GU324291.1

washed, trypsinized and resuspended in freshly prepared 75 mM KCl and incubated for 30 min at 37 °C. They were washed and then fixed in chilled fixative (3:1, methanol: glacial acetic acid) for 20 min at room temperature and centrifuged at 200 g for 10 min. The pellets were resuspended in 5 ml of chilled fixative for another 10 min and then centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides and stained with 2 % giemsa for 5–6 min. The glass slides were rinsed and observed under oil immersion (1,000×) using a compound microscope (Nikon, Microphot, FXA, Japan).

Results

Isolation of spermatogonial and sertoli cells

Greatest enrichment of putative spermatogonial stem cell was seen in the non-adherent fraction from flasks incubated overnight (DSA-coated) (Fig. 1a). Overnight culture in DSA-coated flasks resulted in significant depletion of sertoli cells from the nonadherent fraction. Viability of unattached cells was significantly decreased following overnight incubation in DSA flasks, compared with control (90 %). The adhering cells population obtained from DSA-lectin isolation proliferated and created a monolayer of cells. They had an irregular outline with a granular appearance. In general, the Sertoli cells lost their long cytoplasmatic extensions after enzymatic digestion and were transformed into round cells with crenated edges [12]. After 1 week of culture, they developed extensions, flattened and make contact with other cells. Contamination of Sertoli cell preparation into other cell types was also observed. On the basis of their unique morphological characteristics, no germ cell was evident in the preparation from the time of plating onward. As shown 95 % of the cells in the monolayer were negative for the alkaline phosphatase staining.

Percoll isolation

The highest yield of putative spermatogonial stem cells was found at the interface of densities at 36 % and 34 %. Viability did not differ from that of input cells at any interface. However, viability of cells decreased after percoll isolation in all the interfaces (65 % to 70 %) as compared to control (90 %). Number of colonies formed was significantly higher in percoll isolated group as compared to control, whereas no comparable difference in diameter of colonies.

Colony assay in SSCs co-cultured with sertoli cell groups

To determine effect of Sertoli cell feeder layer on isolated putative SSCs, 10⁶ testis cells/ml were placed in individual wells and examined periodically. There were distinct morphological differences of colonies in presence or absence of Sertoli cell feeder layer. The average number of the colonies in the co cultured group was significantly higher than in the other groups. In the co-cultured group, the colonies appeared earlier (from the day 7 of co-culturing with Sertoli cell feeder layer) compared to the colony formation time in the other groups (on the day 10 or after passage). Also, GDNF 50 ng/ml was shown to be best factor for in vitro colonization of the buffalo SSCs (Fig. 1b and c) as the maximum number of colonies were able to survive under this group when compared with control and other treatment groups. In presence of 50 ng/ml GDNF the diameter of colonies were larger and cells retain proliferation and self renewal capacity than other groups.

Expression of specific markers for putative SSC from prepubertal buffalo testis

Buffalo spermatogonia collected from prepubertal testis were cultured in vitro. After 5–7 days of culture three-dimensional

Fig. 1 Buffalo type-A spermatogonial colonies: (a) Initiation of colony formation (b) Morphology of a spermatogonial-derived colony (c) expansion of SSC colony (d) expression of pluripotency markers i.e. Alkaline phosphatase

colonies were observed. To characterize these colonies, immunocytochemistry was used. The cell types, with spherical outline and two or three eccentrically placed nucleoli, created a colony after proliferation during the first week or immediately after subculture. The colonies were defined as blob-like

structures. These resulted colonies had shown AP activity (Fig. 1d). Furthermore, DBA, OCT-4, CD9 and SSEA-1 which are molecular markers of SSCs, was detected in these colonies (Fig. 2). Vimentin, which is a molecular marker of Sertoli cells, was observed in the feeder monolayer cells and

Fig. 2 Buffalo type-A spermatogonial colonies: (a, d) OCT 4 was detected within the colony cells, (b, e) Positive expression of CD-9, (c, f) colonies showing SSC specific marker i.e. Dolichos biflorus agglutinin, (g, h) SSEA-1 was also seen in the colonies

Fig. 3 Sertoli cells. **a** The cell population, obtained from DSA-lectin isolation, proliferated and created a monolayer of cells after 1 week of cultivation. **b** Vimentin was detected in the feeder monolayer cells (**c**) Merge (Vimentin + Hoechst) (**d**) Hoechst staining of sertoli cells (**e**) the control group, under similar conditions except for the removal of the primary antibody (**f**) the monolayer cells showed no alkaline phosphatase reactivity

the majority of the cells in the monolayer were positive of vimentin (Fig. 3).

Gene expression analysis

RT-PCR demonstrated that the putative SSC grown on sertoli cell feeder layer, express a subset of those genes expressed in the testis, as shown (Fig. 4), which includes the pluripotency markers OCT4 (octamer-binding transcription factor-4), REX-1 and NANOG. SOX2 (SRY-box 2) expression, however, could not be detected in either the isolated SSCs or the testis sample. Apart from that, expression of the SSC specific genes PLZF and VASA was observed in the cells. However, expression of the markers c-Myc was not detected.

Spectral karyotyping

It has been observed that the putative SSC has a normal karyotype and there were no indications of other cytogenetic abnormalities observed (Fig. 5).

Discussion

Our results successfully demonstrate the in vitro culture conditions to support long term maintenance and self renewal of SSCs like cells from prepubertal buffalo calf testis

which stay karyotypically normal and showed expression of SSC specific markers.

We minimized unknown characteristics in the culture system. First, enriched cell populations of SSCs were selected to eliminate the effect of testicular somatic cells or differentiating germ cells. Second, mitotically inactive sertoli cell feeder layer was used. At the end, a testis cell population with a high concentration of SSCs was identified, and a culture condition that maintained the pluripotency of SSCs was established. Under these culture conditions, SSCs colonies were maintained in vitro without significant loss of stemness activity.

The donor bulls used in this study were 3–7 month of age, a development stage in which gonocytes or spermatogonial stem cells are the abundant and present within the testis. Culture of bovine putative SSCs on sertoli cell feeder monolayer for 1 week resulted in a significant increase in round cell colonies. Colonies probably start to develop when spermatogonia and Sertoli cells make contact, apparently creating a microenvironment that favours their development. SSC might need specific micro contacts with niche-offering Sertoli cells during culture [2]. Physical contact and secreted growth factors and cytokines affect the survival of spermatogonial stem cells and provide a suitable microenvironment for proliferation and colonies formation. Previous studies have shown that Sertoli cells monolayer support germ cells proliferation and differentiation [7]. Better results have been obtained with co-cultures of Sertoli cells with

Fig. 4 Expression of transcription based markers in spermatogonial cell: M=100 bp, Lane 1=OCT4, Lane 2=-ve PCR, Lane 3=NANOG, Lane 4=-ve PCR, Lane 5=REX1, Lane 6=-ve PCR, Lane 7=VASA,

Lane 8=-ve PCR, Lane 9=PLZF, Lane 10=-ve PCR, Lane 11=GAPDH and Lane 12=-ve PCR

Fig. 5 Karyotyping of putative SSC

mouse and bovine spermatogonia. These colonies starts degenerating after 1 week in absence of GDNF, the main growth factor which is responsible for SSC self renewal. Sertoli cells feeder layer must be secreting some factors along with GDNF which are supporting the SSC colonies in vitro. Mouse spermatogonial stem cells have been shown to decline during the first 7 days of culture on feeder cells compared to the starting cell population [18]. However, the addition of GDNF to the medium enhanced spermatogonial stem cell numbers in these cultures, implicating GDNF as a factor for spermatogonial stem cell maintenance [19]. Other studies have also indicated the proliferative effects of GDNF on undifferentiated spermatogonia in mouse [12]. After addition of GDNF to culture system, the self maintenance and proliferation of SSC colonies increased. In the presence of GDNF in culture system of SSC on feeder layer, the colonies were able to be maintained in vitro for long period. This shows that GDNF was responsible factor for self maintenance and pluripotency of buffalo SSC in vitro.

DBA has been used as a marker for prespermatogonia, the precursors of bovine spermatogonia present until the onset of spermatogenesis at week 30 of age [8]. Due to the lack of its binding to mouse germ cells, it has been used as marker of bull donor germ cells in mouse testes after transplantation [13].

For confirmation of the presence of spermatogonial stem cells in resulted colonies in addition to alkaline phosphatase activity assessment, CD9 and OCT-4 were traced in the colony cells as well. OCT4 levels are tightly linked to cell fate. In the inner cell mass and ES cells, relatively high levels are required to maintain pluripotency [20], although over expression in ES cells can also lead to their differentiation and similarly over expression in adult tissues can lead to dysplasia [11]. OCT4 has also been found in a mounting

number of adult stem cell types [30], although it was recently shown to be dispensable in several somatic stem cell types, possibly because it is expressed at very low levels in these cells [16]. Like adult somatic stem cells, SSCs in the testis and in vitro expression OCT4 observed at relatively low levels compared with ES cells [14, 15]. Despite the overall low levels of OCT4 in SSCs, we found that OCT4 is required for SSC maintenance. Thus, SSCs, a germ cell and adult stem cell type, may have properties reflecting their unique position at the developmental borderline between embryonic and adult stem cells: a low level of OCT4 required for SSC maintenance and the capacity to be reprogrammed to a pluripotent ES-like state expressing high levels of OCT4 through a change in culture conditions [10, 28, 29].

Whereas for confirmation of the presence and purification of Sertoli cells as feeder cells, in addition to alkaline phosphatase activity assessment, specific marker detection was carried out by immunocytochemistry using anti-Vimentin antibody. Feeder-monolayer did not show alkaline phosphatase activity but vimentin activity. Vimentin is a cytoskeletal protein usually found in the epithelial cells; this protein is also a marker for Sertoli cells [1, 27] and is strictly localized at the perinuclear region of the cells [17, 23]. This finding is in agreement with the reports by Scarpino et al., [27] as well as Anway et al., [2003]. Number of the colonies in all groups declined at the 4 weeks.

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Conflict of interests The authors declare that they have no conflict of interest.

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