Derivation, enrichment and characterization of goat (Capra hircus) spermatogonial stem cells from pre-pubertal testes

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ABSTRACT
Isolation of goat spermatogonial stem cells (gSSC) from pre-pubertal testes (3 to 6 months of age) by double enzymatic digestion is reported. The isolated cells were further enriched for spermatogonial stem cell population by filtration through 80- and 60-µm nylon mesh filters, followed by differential plating on DSA-Lectin coated dishes. After overnight incubation, the unattached cells (putative gSSCs) were cultured on sertoli cell feeder layers in SSC medium, composed of DMEM supplemented with 10% FBS, GDNF (Glial cell line derived neurotrophic factor, 40 ng mL^{-1}), bFGF (Basic fibroblast growth factor, 10 ng mL^{-1}) and EGF (Epidermal growth factor, 10 ng mL^{-1}). After 10-15 days, the putative SSCs started to develop and the cultures were maintained till the colonies stopped growing. The colonies were characterized for their affinity to Dolichos biflorus Agglutinin (DBA), alkaline phosphatase (AP) activity and various spermatogonial stem cell markers (PLZF, THY1, UCHL1, BCL6B and ID4). The developed colonies were positive for DBA, AP and all other markers, which is an indication of their being spermatogonial stem cells. In the present study, goat SSCs have been successfully isolated and characterized and the culture conditions will be improved further for developing more efficient and long term in vitro spermatogonial stem cell culture system in goat.

Key words: Goat spermatogonial stem cells, Testes, Double digestion, Enrichment, Characterization, RT-PCR.

INTRODUCTION
In the male gonad, spermatogonial stem cells (SSCs) reside on the basement membrane of seminiferous tubules. They are able to self-renew and generate a large number of differentiated germ cells finally culminating in sperm formation. The differentiation pathway has remained a scientific curiosity for a long time and various molecular mechanisms have been proposed to be involved in their differentiation to the male gamete. However, a critical balance between these two processes of self renewal and differentiation is deemed essential for the continued production of spermatozoa during the lifetime of an organism. The SSC niche, comprising of adjacent somatic cells, seminiferous tubule epithelium, sertoli and leydig cells, is thought to provide this balance in vivo. The niche determines the fate by providing various crucial factors including physical support, nutrients, hormonal and paracrine signals, all of which are essential for successful spermatogenesis. The mechanisms for regulation of this process are yet to be determined for their potential role in skewing the balance towards spermatogenesis which could provide a treatment for infertility due to oligospermia. The recent interest also lies in use of SSCs for improving the fertility of animals by repopulating recipient testes by SSC transplantation, leading to restoration of spermatogenesis. For example, after depletion of endogenous spermatogenesis in the testes of infertile or low genetic merit animals, repopulation by SSC transplantation from animals of high genetic merit or high fertility could be a way out for rapid propagation of elite germplasm. A number of studies have reported complete spermatogenesis and offspring production following germ cell transplantation into goat testes (Honaramooz et al. 2003a, b). The developmental of culture systems for propagation of SSCs in vitro would make them suitable candidates for targeted genetic modification, as SSCs are capable of directly contributing to the germ lineage and next generation of the animal. In addition to development of efficient isolation and culture systems, there is an urgent need to identify a unique marker or a battery of markers for further enrichment and purification of SSC fraction from other testicular cells, keeping in view their low availability in the male gonad (0.03% in mouse testes) (Tagelenbosch and de Rooij 1993). Currently gonocytes and SSCs are presumed to share a similar set of markers like PLZF, UCHL1, BCL6B, THY1 and ID4 making the two cell populations almost inseparable. In the present study, isolation and enrichment of SSC population

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from goat testes of Indian origin and their further characterization for cell markers so as to establish their identity are reported.

MATERIALS AND METHODS
The chemicals were purchased from Sigma Chemical (St Louis, MO, USA), unless otherwise mentioned. Plastic ware was purchased from Nunc (Roskilde, Denmark) and the nylon mesh filters from Millipore (Bedford, MA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA) and Dolichos biflorus agglutinin (DBA) was purchased from Vector Laboratories (Burlingame, CA, USA).

Isolation and enrichment of goat spermatogonial stem cells
Goat testes were collected immediately after the slaughter of pre-pubertal bucks (3 to 6 months of age) from a local abattoir. The testes were transported to the laboratory within 1 h in normal saline solution fortified with antibiotics (gentamicin 50 µg mL⁻¹), where they were again washed 3 to 4 times with normal saline containing 400 IU mL⁻¹ penicillin and 500 µg mL⁻¹ streptomycin. After this tunica albuginea was removed with a sharp surgical blade and 4–5 g of the exposed seminiferous tubules were isolated and minced in Dulbecco’s modified Eagle’s medium (DMEM), containing 50 µg mL⁻¹ gentamicin sulfate. The minced tissue was subjected to enzymatic digestion, as per the previously described protocol (Izadyar et al. 2002) with some modifications. Briefly, tissues were first incubated with 1 mg mL⁻¹ collagenase, 1 mg mL⁻¹ hyaluronidase Type II, 5 µg mL⁻¹ DNase and 1 mg mL⁻¹ trypsin at 37°C in a shaker incubator for 45 minutes. The supernatant was discarded and the pellet, obtained by centrifugation at 40 g for 3 min, was treated with 1 mg mL⁻¹ collagenase, 1 mg mL⁻¹ hyaluronidase Type II and 5 µg mL⁻¹ DNase, in a shaker incubator for 30 min. This was followed by centrifugation at 50 g for 2 min. The supernatant, which was presumed to contain spermatogonial stem cells (SSCs), sertoli cells, myeloid cells and other contaminating cells of the seminiferous tubular tissue, was filtered through 80- and then 60-µm nylon mesh filters to enrich the SSC population. The supernatant was seeded onto DSA-Lectin coated dishes, prepared by coating 35 mm Petri dishes with lectin from Datura stramonium agglutinin (5 µg mL⁻¹ in DPBS) and blocked by addition of 0.6% BSA for 2 h. The cultures were incubated overnight at 37°C in a CO₂ incubator. The still unattached cells in the medium were collected by centrifugation at 100 g for 5 min and seeded onto sertoli cell feeder layers in 35 mm Petri dishes.

Preparation of feeder layer
Sertoli cells, which constitute the major fraction of DSA-Lectin bound cell population, were cultured for 2–3 days at 37°C in 5% CO₂ incubator in the culture medium composed of DMEM supplemented with 10% FBS, 4M L-glutamine, 1% (v/v) non-essential amino acids and 50 µg mL⁻¹ gentamicin sulphate. The feeder cells were sub-cultured at 80% confluence by trypsinisation and were propagated in 25 cm² culture flasks. At 70% confluence, the sertoli cells were again trypsinised, seeded in 35 mm Petri dishes for 16 h and treated with 10 µg mL⁻¹ mitomycin C for 3 h. The cultures were washed thrice with DPBS, to remove any traces of mitomycin C, and were used as feeder cells for SSC culture.

In vitro culture of spermatogonial stem cells
Cells enriched by differential plating were seeded on the sertoli cell feeder layers and cultured in SSC medium (DMEM supplemented 10% FBS, 40 ng mL⁻¹ GDNF, 10 ng mL⁻¹ bFGF and 10 ng mL⁻¹ EGF) at 37°C in a CO₂ incubator for 10-15 days, till putative SSC colonies were formed. The medium was replaced every third day with fresh SSC medium during the culture period.

Characterisation of spermatogonial stem cells
Alkaline phosphatase activity
Alkaline phosphatase staining was performed using a commercially available kit (catalogue no. 86C; Sigma Chemical), as per the kit instructions. The medium was removed from the cultures, and were washed thrice with DPBS. This was followed by fixation with citrate–acetone–formaldehyde fixative solution for 1 min and three washings with deionised water. After that alkaline dye was added and the cultures were incubated for 15 min at room temperature. The dishes were rinsed three times with deionised water and counterstained with neutral red stain for 1–2 min. The colonies were washed several times to remove the extra neutral red stain. The red coloured colonies were considered positive for alkaline phosphatase activity and were presumed to be putative SSCs.

The expression of DBA in SSC colonies was examined by immunofluorescence staining. The colonies were fixed in 4% paraformaldehyde in DPBS for 30 min and permeabilised by treatment with 0.1% Triton X-100 in DPBS for 30 min. The cells were incubated with blocking solution (5% BSA) for 30 min and then with Fluoresceine-labelled DBA (1:100) for 1 h at room temperature. The cells were washed 3 times with DPBS followed by examination under a fluorescence microscope (Nikon, Tokyo, Japan).

Detection of SSC-specific transcription factors by RT-PCR
Spermatogonial stem cell colonies were detached from the feeder layer by manual cutting with a Microblade under 20X zoom stereomicroscope. The colony clumps were trypsinised, seeded in 35 mm Petri dishes for 16 h and treated with 10 µg mL⁻¹ mitomycin C for 3 h. The still unattached cells in the medium were collected by centrifugation at 100 g for 5 min and seeded onto sertoli cell feeder layers in 35 mm Petri dishes.

Total RNA was extracted using RNeasy-Micro Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The extracted total RNA was subjected to DNase treatment to remove any contaminating genomic DNA. The purity and concentration of RNA was checked by Nanoquant (Teccan, Salzburg, Austria). Only those RNA samples with 260/280 absorbance ratio > 1.9 were used for cDNA synthesis, employing RevertAid First Strand cDNA Synthesis.
RESULTS AND DISCUSSION

Isolation and enrichment of spermatogonial stem cells: An attempt was made to standardize a protocol for isolation and maintenance of putative goat spermatogonial stem cells.

Table 1: Primers used for goat spermatogonial stem cell characterization.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5ˈ–3ˈ)</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL6B</td>
<td>Forward: GCCACCACCTTTAATTTCTCAC [ID4] Reverse: GAAATACGCTTCCAGTCTC</td>
<td>58°C</td>
<td>162</td>
<td>XM_005693476.1</td>
</tr>
<tr>
<td>ID4</td>
<td>Forward: TGTCGCTAGTTCATGCTCG [UCHL1] Reverse: AGAAAGTGGTTCTATGGCCAAGAG</td>
<td>56°C</td>
<td>102</td>
<td>XR_139666.2</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Forward: GATAAGAGCTTTACCCTCCAACC [PLZF] Reverse: GCCCTAACCTTACAGACACAACC</td>
<td>58°C</td>
<td>165</td>
<td>XM_005681551.1</td>
</tr>
<tr>
<td>PLZF</td>
<td>Forward: GCACAGGCCAGCATCTACCTC Reverse: TGACGAGCCTCAGACAGGTC</td>
<td>56°C</td>
<td>118</td>
<td>JX047313.1</td>
</tr>
<tr>
<td>THY 1</td>
<td>Forward: TCAGCAGTCATCCAGCTC Reverse: TGAAATAGGAAAGGTTGGAAGG</td>
<td>56°C</td>
<td>124</td>
<td>XM_005689553.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: TCAAGGAAGTGTTGGAAGCAG Reverse: CCCAGCATCGAAGGTGAAGA</td>
<td>56°C</td>
<td>157</td>
<td>Shah et al. 2015</td>
</tr>
</tbody>
</table>

The pre-pubertal testes from 3-6 m old bucks were used for the purpose, as the testes at this age are reported to harbour an abundant population of gonocytes or SSCs (Aponte et al. 2006). A number of different enrichment strategies among the species have been used but the greatest enrichment of putative spermatogonial stem cells has been reported in non-adherent fraction from overnight incubated DSA-Lectin coated 35 mm culture dishes. These studies support our experimental design to use pre-pubertal testes for gSSC isolation and further enrich the cell population on DSA-Lectin coated dishes. Since, most of the somatic cells get adhered to DSA-Lectin, a significant depletion of sertoli cells and other somatic cells from the non-adherent fraction is presumed. For maintenance of the isolated SSCs in culture, we used sertoli cell feeder layer as sertoli cells have been reported to support the growth and colony formation of both the bovine and human SSCs (Oatley et al. 2004; Aponte et al. 2006; Sousa et al. 2002) and thus could be expected to do the same role in goat SSCs. The ability to form colonies has been attributed to be the result of complex interactions between SSCs and Sertoli cells (Oatley et al. 2004; Aponte et al. 2006). We observed a three-dimensional colony formation within 15-20 days on sertoli cell feeder layer culture (Fig. 1). This duration was similar to that reported for mice (Kanat et al. 2003), pigs (Kuijk et al. 2009), cattle (Aponte et al. 2008), buffalo (Kala et al. 2012) as well as goat (Pramod and Mitra 2014). The colonies probably start to develop when spermatogonia and sertoli cells make contact, apparently creating a microenvironment that favours their development. Thus, the physical contact between spermatogonial and sertoli cells and the secreted growth factors and cytokines provide a suitable microenvironment for SSC proliferation and colony formation. To complement sertoli cell support, addition of various growth factors like GDNF, bFGF and EGF in SSC culture medium has been reported across the species. GDNF has been reported to play an important role in the maintenance and self-renewal of mouse (Kanatsu et al. 2003; Kubota et al. 2006) and thus could be expected to do the same role in goat SSCs. The colonies have been reported to be the result of complex interactions between SSCs and Sertoli cells (Oatley et al. 2004; Aponte et al. 2006).
Characterization of spermatogonial stem cells: These putative SSC colonies expressed high alkaline phosphatase activity (Fig. 2) which is also expressed by buffalo (Kala et al. 2012, Kadam et al. 2012) and goat (Wang et al. 2014) SSCs. Furthermore DBA, which is a specific marker for gonocytes and type A spermatogonia from pre-pubertal bovine and buffalo testes (Herrid et al. 2007, Goel et al. 2010, 2011, Fujihara et al. 2011) was also expressed (Fig. 3), demonstrating their similarity with bovine and buffalo SSCs. The expression of markers such as PLZF, THY1, UCHL1, BCL6B and ID4 (Fig. 4) further support that these cells are putative SSCs.

Among the transcription factors, PLZF expression has been reported in gonocytes and undifferentiated spermatagonia and has been shown to be essential for spermatogonial stem cell maintenance and self-renewal in mouse (Luo et al. 2006), goat (Pramod and Mitra 2014) cattle (Reding et al. 2010) and buffalo (Kadam et al. 2012). THY1 has been shown to be a unique surface marker of SSCs in mice (Kubota et al. 2004b), cattle (Reding et al. 2010), buffalo (Kadam et al. 2012) and goat (Abbasi et al. 2013), while UCHL1 is expressed in pre-meiotic male germ cells. Its expression has been reported in pre-pubertal testes from pigs, cattle and buffalo (Luo et al. 2006, Herrid et al. 2007, Goel et al. 2010).
et al. 2010). ID4 has also been reported to be unique marker for single spermatogonia in murine species (Oatley et al. 2011). The expression of all these markers provides sufficient evidence that the putative colonies are indeed spermatogonial stem cells.

In summary, we report the isolation and enrichment strategy for goat spermatogonial stem cells from pre-pubertal testes and their maintenance under in vitro culture conditions. We further report a panel of markers that could be used for their characterization and further enrichment.

REFERENCES


