



Homologous transplantation of fluorescently labelled enriched buffalo (*Bubalus bubalis*) spermatogonial stem cells to prepubertal recipients

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ABSTRACT

Spermatogonial stem cell transplantation provides a unique opportunity to study the biology of spermatogenesis and also offers an alternative approach for genetic modification in large animals. The present study aimed to extend this technique to the water buffalo. Spermatogonial stem cells (SSCs) were isolated from prepubertal buffalo testes (3–6 months of age) using two-step enzymatic digestion method and enriched by differential plating and Percoll density gradient centrifugation. The enriched SSCs expressed numerous spermatogonial transcriptional markers, viz. *ID4*, *THY1*, *BCL6B*, *UCHL1*, *ETV5* and *REX1* which confirmed their bonafide SSC identity. Subsequently, the enriched SSCs were labelled with a fluorescent dye PKH26 and transplanted into buffalo calves under ultrasound guidance. The recipient testes were recovered after 7–8 weeks by castration and their fluorescence microscope-based examination exhibited the persistence and localization of the fluorescent donor cells within the recipient seminiferous tubules. Further validation was done by the flow cytometric evaluation of PKH26 labeled donor cells among those isolated by two-step enzymatic digestion of recipient testicular parenchyma. In conclusion, we demonstrated the feasibility of SSC transplantation technique in the water buffalo.

Keywords: Buffalo, PKH26, Spermatogonial stem cells, Transplantation

Spermatogenesis is a highly orchestrated sequence of events in which spermatogonial stem cells (SSCs) steadily replicate and generates millions of spermatozoa every day throughout the reproductive life of the male animals. Spermatogonial stem cell transplantation (SSC transplantation), a technique first introduced in mice (Brinster and Averbach 1994, Brinster and Zimmermann 1994) is based on injection of SSCs from fertile donors to the infertile recipients culminating in donor-derived sperm production. The donors SSCs when transplanted into recipient testes retain capacity to colonize the stem cell niches present in the host seminiferous tubules and restore spermatogenesis. After effective implementation in mouse model, SSC transplantation has been successfully adapted in numerous species including farm animals where donor SSCs derived sperm production following transplantation had been reported in goat (Honaramooz *et al.* 2008, Zeng *et al.* 2012), pigs (Zeng *et al.* 2013), cattle (Izadyar *et al.* 2003, Stockwell *et al.* 2009) and camel (Herrid *et al.* 2019). Apart from exhibiting tremendous potential for restoration of fertility in infertile/sub-fertile recipients, SSC transplantation has emerged as an efficient method for generating transgenic animals. The unique property of SSCs of being the only adult stem cells capable of transmitting their genetic information to the next generation can be

exploited for transgenesis in farm animals. The current methods for generating transgenic farm animals are pronuclear microinjection and somatic cell nuclear transfer (SCNT), although these methods are costly and inefficient. Moreover, the offspring produced by SCNT often suffers from developmental abnormalities (Mehta *et al.* 2017).

SSCs can be isolated and cultured with ease, and when transplanted after their genetic modification, can produce transgenic spermatozoa. This technique has been successfully applied to produce transgenic mice (Nagano *et al.* 2000, Kanatsu-Shinohara *et al.* 2004), rats (Hamra *et al.* 2002) and goat (Honaramooz *et al.* 2003a). Furthermore, transgenic sperm production by the genetically modified SSCs in goats (Honaramooz *et al.* 2008, Zeng *et al.* 2012) and pigs (Zeng *et al.* 2013) ensures the promising applications of the technique in this field.

Buffalo has been considered as lifeline to the rural economy due to its enormous contribution in milk, food and draught power generation (Srivastava and Kumaresan 2014). Being the second largest milk contributor in the world and high fat and protein content of its milk, it is a suitable model for biopharmaceutical protein production using its mammary gland as bioreactor (Mehta *et al.* 2018, 2019). SSC transplantation could play a crucial role in genetic improvement in the water buffalo and generate transgenic buffaloes producing medicinal value proteins in their milk. In earlier studies, bubaline SSCs have been

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xenotransplanted in nude mice to confirm their stem cell potential (Mahla *et al.* 2012, Yu *et al.* 2014), but their homologous transplantation in buffalo has remained unexplored till date.

With this backdrop, the present study was undertaken to optimize homologous SSC transplantation in the water buffalo using fluorescently labelled enriched SSCs. Its success will lay the foundation of generating transgenic buffaloes by genetic modification of male germline stem cells.

MATERIALS AND METHODS

The present study was carried out at the Embryo Biotechnology Laboratory, Animal Biotechnology Centre and Livestock Research Centre, ICAR-National Dairy Research Institute, Karnal, India. Unless mentioned otherwise, chemicals were purchased from Sigma Chemical (St Louis, MO, USA). Plasticwares were procured from Nunc (Roskilde, Denmark) and nylon mesh filters from Millipore (Bedford, MA, USA).

Isolation and enrichment of SSCs: Prepubertal buffalo (3–6 months of age) testes were collected from a local abattoir. Isolation and enrichment of putative SSCs were performed as described in our earlier reports (Sharma *et al.* 2016, Sharma *et al.* 2019a). Briefly, minced seminiferous tissue was subjected to a two-step enzymatic digestion followed by filtration through 80- and then 60- μ m nylon mesh filters. In order to remove the contaminating somatic cells, the filtered cells were seeded onto *Datura stramonium* agglutinin (DSA) lectin coated 35 mm culture dishes at a density of 2×10^5 cells/cm² and were incubated overnight at 37°C in a CO₂ incubator (5% CO₂ in air). Subsequently, the enriched cells in suspension were harvested and subjected to Percoll density gradient centrifugation as described previously (Kadam *et al.* 2013). The enriched SSCs found in the interface between 28, 30 and 32% Percoll suspensions were collected and cultured in DMEM supplemented with 10% FBS at 37°C.

Characterization of putative SSCs by reverse

transcriptase (RT) PCR: The RT-PCR based characterization of enriched SSCs was performed as described previously (Sharma *et al.* 2019b). Briefly, total RNA was extracted using RNAqueous-Micro Kit (Ambion, Austin, TX, USA) and its concentration and purity was determined by Nanoquant (Teccan, Salzburg, Austria). The cDNA was synthesized using Superscript III, first strand cDNA synthesis kit (Invitrogen). The primer sequence, annealing temperature, amplified product length, and GenBank accession numbers of the original sequences are given in Table 1. The RT-PCR reactions were set up in a final volume of 25 μ L having 12.5 μ L DreamTaq Green PCR Master Mix (2 $\times$) (Thermo Scientific, USA), 0.5 μ L of 10 μ M of each primer, and 2 μ L cDNA (200 ng). The thermal cycling conditions were 94°C for 3 min, followed by a cycling program of 94°C for 30 sec, X°C (annealing temperature as mentioned for each primer pair in Table 1) for 30 sec and 72°C for 30 sec for 35 cycles, followed by final extension at 72°C for 10 min. GAPDH was used as internal control. Non-template controls and reverse transcriptase-negative controls were also set for every reaction. The PCR products were visualized on 2% agarose gel for the specific products.

Labelling of enriched SSCs with PKH 26 fluorescent dye: To visualize the transplanted enriched SSCs in recipient buffalo testes, the donor cells were labelled with Red Fluorescent Cell Linker PKH26 (Sigma) according to the manufacturer's instructions. Briefly, 10 million enriched SSCs were washed in DMEM, centrifuged at 400 g for 5 min to form a loose pellete, and re-suspended in diluent C supplied with the kit. Shortly before labelling, 4×10^{-6} M PKH26 dye was prepared using the diluent C and gently mixed with the cells. After 3 min of incubation, the reaction was terminated by adding equal volume of FBS, and the cells were washed thrice with DMEM. After the last wash, the cells were re-suspended in DMEM + 5% FBS for the transfer.

Recipient preparation and transplantation: Murrah buffalo calves (n=3; age 13–15 months) were given epidural

Table 1. Primers used for gene expression studies

Gene	Primer sequence (5'–3')	Annealing temperature (°C)	Product length (bp)	Accession no.
<i>BCL6B</i>	Forward: GCCACCACCTTTAATTTCTCAC Reverse: GAAATCAGGCTTCCAGTCTC	58	162	XM_005693476.1
<i>ID 4</i>	Forward: TGTCAGTCTGAGTTTCATGTCTG Reverse: AGAAAGTGTTTCATTGCCAAGAG	56	102	XR_139666.2
<i>UCHL 1</i>	Forward: GATAAGCACTTACCCTCAACC Reverse: GCCTTAACTTACAGACACAAACC	58	165	XM_005681551.1
<i>THY 1</i>	Forward: TGCTAACAGTCTTACAGGTG Reverse: GGCTGAACCTCATACTGAATGG	57	131	BC104530.1
<i>REX 1</i>	Forward: CAGCTGCCATAGGCTCTACC Reverse: GTCTGGCCTGACAAAGGTGT	55	239	XM_001255545
<i>ETV 5</i>	Forward: CTGCCAGTCTTACATGAGAG Reverse: TTTCACCTTGCCTTCCAGTC	58	126	NM_001192834.1
<i>GAPDH</i>	Forward: TCAAGAAGGTGGTGAAGCAG Reverse: CCCAGCATCGAAGGTAGAAG	56	157	GU324291.1

anaesthesia (2% lignocaine; Lignocaine HCl Injection BP, Pfizer Australia Pvt Ltd) and restrained in lateral recumbency. Transplantation of SSCs was performed under ultrasound guidance (Aloka Prosound 2, Hitachi Aloka Medical Ltd., USA) using a 6.5 MHz rectal linear probe (Aloka UST-5820-5, Aloka). The probe was aligned along the length of one of the testes receiving donor cells. The rete testis was visualized and a volume of 2 mL media containing 10 million fluorescently labelled cells was slowly released by using a 22G needle attached syringe. Post-transplantation veterinary care was given as per the standard procedure.

Analysis of the recipient testes: Testes receiving germ cell transplantation were recovered by castration after 7 to 8 weeks of transplantation. The presence and distribution of the PKH26 labelled donor cells was examined as described previously (Honaramooz *et al.* 2002, 2003b). Briefly, representative samples of seminiferous tubules were collected by scraping a surgical blade across the longitudinally sectioned testes and examined under fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan) at 100–200 $\times$ magnification. Seminiferous tubules from slaughter house testes were used as negative control. Then, randomly collected testicular parenchyma samples from recipient testes were subjected to two-step enzymatic digestion and the presence of PKH26 labelled donor cells was examined by FACS (MoFlo XDP, Beckman Coulter Inc., USA) equipped with a 15 mW air cooled 488 argon-ion laser. The emission fluorescence was collected using a 575/25 nm band pass (BP) filter and the positive cell population expressing PKH26 was assessed. Unstained cells were used as negative control to determine the PMT voltage and to set the population gate. For each sample, at least 10,000 events were acquired within the singlet gate of forward scatter v/s side scatter. Data were analysed using summit software.

RESULTS AND DISCUSSION

The present study demonstrates successful transplantation of fluorescently labelled enriched buffalo SSCs to the homologous recipients. Prepubertal buffalo (3–6 months old) testes were used for the isolation of SSCs as they bear an ample number of gonocytes/SSCs at this age (Ahmad *et al.* 2013). Since, SSCs are very rare population in testis (~0.03% in adult mouse testis; Tagelenbosch and de Rooij 1993); and till date, any unique marker has not

been reported for their identification. In current scenario, SSC enrichment is the only practicable way to acquire their sufficient number for use in germ cell transplantation. In earlier studies, the combined use of differential plating and Percoll density gradient centrifugation could generate highly enriched population of buffalo SSCs (Goel *et al.* 2010, Ahmad *et al.* 2013). Therefore, the bubaline SSCs were enriched using differential plating with DSA lectin coated dishes followed by Percoll density gradient centrifugation. Thereafter, the enriched SSCs were subjected to RT-PCR based characterization where the expression of spermatogonial markers, viz. *ID4*, *THY1*, *BCL6B*, *UCHL1*, *ETV5* and *REX1* confirmed their bonafide SSC identity (Supplementary Fig. 1).

In the present study, prepubertal buffalo males were used as recipients because their seminiferous tubules lack multiple layers of differentiating spermatogonia which hinder the access of donor SSCs to their stem cell niches (Shinohara *et al.* 2001). The attenuation of endogenous germs cells prior to the germ cell transplantation has improved the colonization and expansion of donor SSCs in adult rodents (Brinster and Zimmermann 1994, Shinohara *et al.* 2002). Although SSC transplantation has been successful in prepubertal goat (Honaramooz *et al.* 2003a), pig (Zeng *et al.* 2013) and cattle (Herrid *et al.* 2006) without any pre-treatment for depletion of endogenous

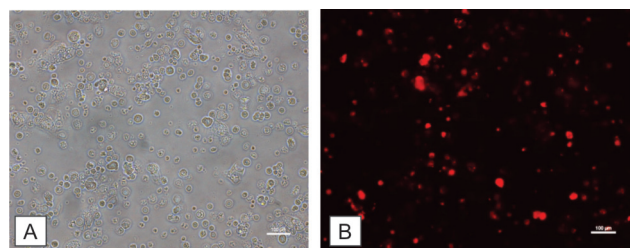


Fig.1. Labelling of enriched SSCs with fluorescent dye PKH26. (A) Bright field, (B) Fluorescence under green light. Magnification 40 $\times$. Scale bar 100 μ m.

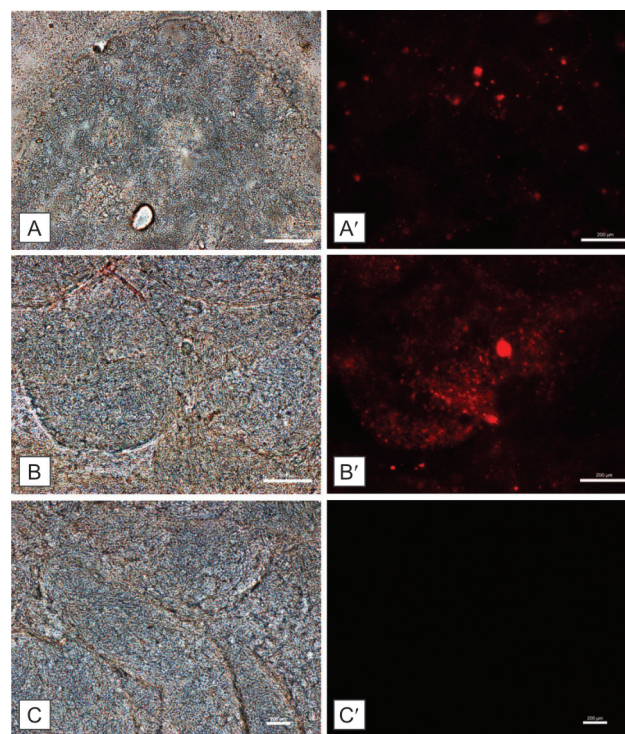


Fig. 2. Evaluation of recipient seminiferous tubules for the presence of PKH26 labelled enriched SSCs after 8 weeks of transplantation (A, A' and B, B'); Light and corresponding fluorescence photomicrographs of dispersed seminiferous tubules illustrating the presence of fluorescent-labelled (PKH26) cells; (C, C') Seminiferous tubules from untreated testes as negative control. Magnification: (A, A': 200 $\times$), (B, B', C, C': 100 $\times$). Scale bar 200 μ m.

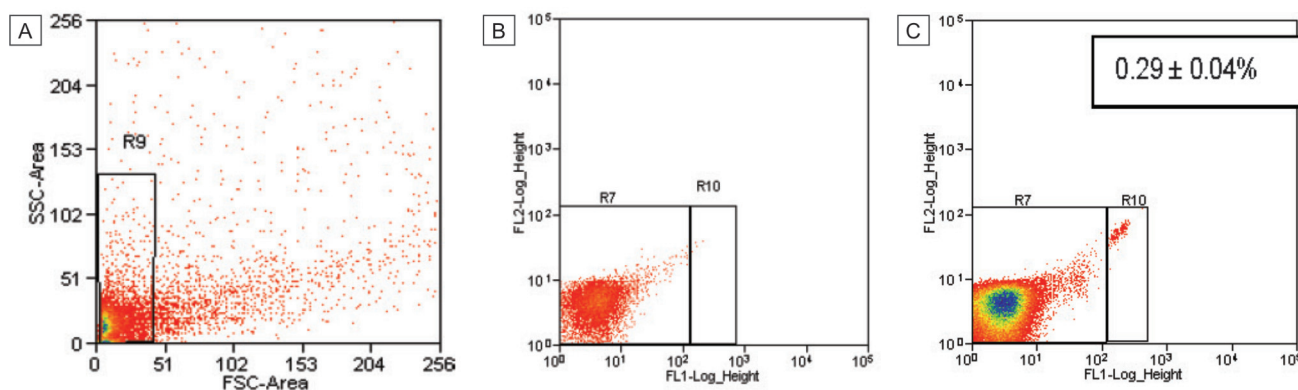


Fig. 3. Flow cytometry based evaluation of PKH26 expression in testicular cells isolated after two-step enzymatic digestion of recipient testicular parenchyma. A) Forward scatter vs Side scatter for the selection of population; B) Control (cells isolated from untreated testes); C) Cells isolated from the testes receiving PKH26 labelled enriched SSCs. Values are mean $\pm$ SEM.

spermatogenesis which indicates that recipient preparation in immature farm animals may not be as significant as in case of adult rodents (Honaramooz and Yang 2011). We have also used the recipient buffalo calves without depletion of their germ cells. However, a fluorescent dye PKH26 was used to label the donor cells to monitor their fate in recipient seminiferous tubules (Fig. 1). Previously, donor SSCs labelled with PKH26 dye have been efficiently tracked for the duration of 1–3 months after transplantation (Honaramooz *et al.* 2002, 2003b; Herrid *et al.* 2006).

The recipient testes receiving enriched SSCs were collected by castration and the seminiferous tubule sections were evaluated under fluorescence microscope which confirmed the persistence of fluorescently labelled donor cells even after 8 weeks of transplantation. The donor cells were observed as clusters/ colony and their localization and distribution was in proximity of basement membrane and throughout the seminiferous epithelium (Fig. 2). The flow cytometric evaluation of testicular cells isolated from recipient's testicular parenchyma using two-step enzymatic digestion further showed that $0.29 \pm 0.04\%$ of the total cells was PKH26⁺ (Fig. 3). Based on these results, it can be inferred that the donor enriched SSCs were beard in recipient testes for long enough to colonize and proliferate which indicate that the buffalo seminiferous tubules could be immunologically tolerant for homologous donor germ cells. Our findings support the concept from earlier studies (Honaramooz *et al.* 2002, 2003a; Herrid *et al.* 2006, Zeng *et al.* 2013) that homologous SSC transplantation is feasible in farm animals without using immunosuppressed or genetically related recipients.

In conclusion, we demonstrated successful transplantation of fluorescently labelled enriched buffalo SSCs in homologous recipients. Our objective was to show the feasibility of SSC transplantation technique in buffalo. The future research will focus on the use of this approach to generate transgenic buffaloes.

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