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Research paper

Transgenic expression of green fluorescent protein in caprine embryos produced through electroporation-aided sperm-mediated gene transfer

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ABSTRACT

Current methods of transgenic animal production are afflicted by low efficiency and high cost. Recently, the electroporation aided sperm-mediated gene transfer (SMGT) emerges as a promising alternative with variable success rate. Among the domestic animal species, the electroporation-aided SMGT is less investigated in goats, except a few reports in which attempts have been made using the auto-uptake method of SMGT. In this study, we report an optimized electroporation condition for SMGT of caprine sperm cells. Results of this study demonstrated that electroporation of caprine sperm cells at 300 V for 200 mS in TALP medium allowed the maximum uptake of foreign DNA with minimum adverse effects on the vital semen parameters viz., progressive motility, viability, and membrane and acrosome integrity. Further, DNA binding assay revealed DNA uptake by 81.3% sperm cells when 1.0 μg of DNA was used under optimum electroporation conditions as compared to 16.5% on simple incubation. The qPCR analysis showed four-fold more ($P < 0.05$) DNA uptake by sperm cells under electroporation than incubation. A similar cleavage rate was observed after IVF using either electroporated (23.20 ± 1.20) or non-electroporated (25.20 ± 2.41) sperm cells suggesting the absence of adverse effect of electroporation on the fertilizing ability. Out of the 116 embryos produced by electroporated sperm, five (4.31%) embryos showed the expression of the foreign gene. In conclusion, our results confirm that using optimized electroporation conditions, the caprine sperm cells can uptake foreign DNA effectively with minimum negative effect on the semen parameters and could produce transgenic embryos.

1. Introduction

Sperm-mediated gene transfer (SMGT), a method of transgenesis, is based on the ability of sperm cells to bind, internalize and transport the exogenous DNA into an oocyte during the process of fertilization (Lavitrano et al., 1989). The SMGT method is contemplated as a viable and cost-effective alternative to produce transgenic domestic animals because it does not require any special skill and costly infrastructure (Lavitrano et al., 2003; Smith, 2004). The conventional method of SMGT, which relies on the auto-uptake of foreign DNA by sperm cells on incubation, is perturbed by poor DNA uptake (Anzar and Buhr, 2006). Further, DNA binding efficiency varies widely ranging from 0.3 to 78% among the sperms of different species (Camaioni et al., 1992; Canovas et al., 2010; García-Vázquez et al., 2011; Zhao et al., 2012). Consequently, the success rate of SMGT also varies in different animal species including chicken (Nakanishi and Iritani, 1993), mouse (Maione et al., 1998), Xenopus (Jonak, 2000), zebrafish (Khoo, 2000), pig (Lavitrano et al., 2002) and bovines (Anzar and Buhr, 2006). In order to improve the DNA uptake efficiency of sperm cells, several strategies have been employed including DNA–liposome complexes (Lai et al., 2001), electroporation (Gagne et al., 1991) and monoclonal antibodies to link foreign DNA to sperm (Chang et al., 2002). Amongst those, electroporation-aided SMGT is considered to be cheaper and more efficient method (Sin et al., 2000; Khan, 2010).

Under the electric field, the contact between sperm cells and DNA molecules is markedly increased. It results in an improvement in the DNA uptake as well as DNA retention by sperm cells (Rieth et al., 2000). Thus, following electroporation, foreign DNA is stably captured by the sperm cells and carried into oocytes during fertilization (Gagne et al., 1991). The efficiency of electroporation-aided DNA uptake is, however, greatly influenced by the field strength, a number of pulses applied and the DNA concentration (Symonds et al., 1994). Although, a number of studies suggested the absence of adverse effects of electroporation on fertilization ability of sperm cells (Tsai et al., 1997; Rieth et al., 2000), contrasting results are observed by others (Gagne et al., 1991; Rieth et al., 2000). Generally, higher the voltage, greater the

Abbreviations: AV, Artificial Vagina; BSA, Bovine Serum Albumin; BSA-FAF, Bovine Serum Albumin-Fatty Acid Free; COC, Cumulus–Oocyte Complex; EGFP, Enhanced Green Fluorescent Protein; FITC-PSA, Fluorescein Isothiocyanate-Pisum Sativum Agglutinin; IVC, *In Vitro* Culture; IVF, *In Vitro* Fertilization; IVM, *In Vitro* Maturation; mSOF, Modified Synthetic Oviduct Fluid; OCM, Oocyte Collection Medium; qPCR, Quantitative Real Time PCR; SMGT, Sperm Mediated Gene Transfer; TALP, Tyrods Albumin Lactate Pyruvate.

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efficiency of SMGT (Muller et al., 1992), however, it caused more deleterious effects on semen parameters (Gandolfi, 1998). Using electroporation-aided SMGT, transgenic offsprings are successfully produced in finfish and shellfish (Tsai, 2000), and also in Rohu fish (Thayanithy et al., 2004).

Goat is considered as an ideal livestock species for transgenesis because of their small body size, shorter gestation period, prolificacy and relatively higher milk protein content (Ebert et al., 1991; Meade et al., 1998). But, only a few SMGT studies, that too involving only the auto-uptake method, are carried out in goats (Wang et al., 2011; Zhao et al., 2012). Further, in domestic livestock species including in goat, attempts of using electroporation-aided SMGT are very limited except the study reporting generation of the transgenic bovine embryo (Rieth et al., 2000). In the present manuscript, we report an optimized electroporation condition for SMGT of caprine sperm cells and its subsequent fertilizing ability using *in vitro* fertilization (IVF) technique. Further, we for the first time, demonstrate the production of transgenic caprine embryo employing electroporation-aided SMGT.

2. Materials and methods

2.1. Preparation of plasmid vector

The eukaryotic GFP expression vector p*IRES2-EGFP* (Clontech, USA) was donated by Dr. Majumdar (Core Scientist NII, New Delhi). p*IRES2-EGFP* contains the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. The plasmid was purified with Endotoxin Free Quanta Maxi Kit (mdi, India) and dissolved in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0). The purified plasmid was linearized with StuI restriction enzyme.

2.2. Experimental animals and collection of semen

Adult bucks ($n = 3$) of 2–3 years age, maintained under identical management conditions at experimental herd under Genome Analysis Lab were included in this study for semen collection. Semen from bucks was collected using commercially available artificial vagina (AV) suitable for smaller ruminants (20 cm length and 5 cm diameter).

2.3. Sperm preparation and electroporation

A 10 μl of fresh semen was added to 0.8 ml of Tyrode's albumin lactate pyruvate (TALP) medium, supplemented with 0.6% bovine serum albumin fatty acid free (BSA-FAF) or 0.8 ml of 0.3 M mannitol, 0.1 mM $MgCl_2$, 0.05 $CaCl_2$ in Gene pulser cuvette with a distance of 4 mm between electrodes. Electroporation was carried out with different combinations of voltage and time constant i.e., 100 V for 200 ms, 300 V for 200 ms, 500 V for 600 μs and 1000 V for 300 μs using an electrical pulse generator (Electrosquare Porator, ECM830, BTX, USA). The effect of electroporation was assessed by comparing vital seminal parameters (e.g., progressive motility, live and dead count (viability), membrane and acrosome integrities of sperm cells) before and after the electroporation.

2.4. Evaluation of seminal parameters

To assess progressive motility, a 10 μl of electroporated sperm sample was placed on a microscopic slide, covered with a coverslip and examined under high power (40×) of the phase contrast microscope. Twenty fields were observed and the average number of motile sperm cells (%) was determined. In order to calculate the live/dead percentage of sperms, Eosin–Nigrosin (Rodríguez-Martínez, 2000) based differential staining was used. Acrosomal integrity was determined by using fluorescein isothiocyanate conjugated pisum sativum agglutinin (FITC-PSA; Sukardi et al., 1997) based staining method. Hypo-osmotic swelling test (HOST) was used to assess the functional integrity of the sperm cell membrane (Jeyendran et al., 1984).

2.5. Assessment of DNA uptake by the sperm using fluorescein labeled plasmid

In order to optimize the concentration of foreign DNA for SMGT and to evaluate the foreign DNA uptake by sperm cells after electroporation, the DNA binding assay was performed using fluorescently labeled plasmid. Three different concentrations (0.5 μg, 1.0 μg, and 1.5 μg) of plasmid were used. An incubation (60 min) only using 1.0 μg plasmid without electroporation was also used in this study.

2.5.1. Labeling of transgene

The linearized plasmid was labeled with fluorescein-12-dUTP (Roche, Mannheim, Germany). Random primed DNA labeling method was used to label the transgene. Briefly, 2 μg of linearized plasmid was denatured for 10 min at 95 °C and quickly chilled in ice. Immediately, the components were added as follows; 1 μl, 0.5 mM of dATP, dCTP and dGTP; 0.65 μl 0.5 mM of dTTP; 0.175 μl 0f 1 mM; 2 μl reaction mix and lastly 1 μl Klenow enzyme. Subsequently, the mixture was incubated for overnight at 37 °C. The reaction was stopped by adding 2 μl, 0.2 M EDTA (pH 8). The labeled DNA was precipitated out with chilled ethanol and was finally resuspended in TE buffer (pH 8).

2.5.2. DNA uptake using labeled transgene

A 50 μl of fresh semen was washed with phosphate buffered saline (PBS) for three times at 800 g for 5 min and then the sperm pellet was resuspended in a small quantity of TALP medium. A 10 μl of sperm suspension of 5×10^6 million sperm was added into electroporation cuvette containing 0.8 ml of TALP medium and specific concentrations of fluorescein-labeled plasmid was also added into it. Electroporation was carried out at 300 V for 200 mS using electric pulse generator. After washing three to four times with PBS, finally the sperm pellet was suspended in PBS. In order to detect the fluorescently labeled sperm, a 10 μl of the sperm suspension was kept under the cover slip and examined using a fluorescent microscope (Leica Microsystems, Germany).

2.6. Estimation of DNA uptake using quantitative real-time PCR (qPCR) analysis

The DNA uptake by sperm cells using electroporation was also assessed with quantitative real-time PCR (qPCR). The semen samples were collected from three bucks. For each buck, semen samples collected on four different occasions were subjected to SMGT independently. The optimized concentration of 1 μg linearized plasmid was used for electroporation of sperm cells using 300 V for 200 mS. Following electroporation, both the test (i.e., electroporated) and control (i.e., non-electroporated) samples were subjected to centrifugation at 500 *g* for 10 min. The supernatants (referred as Sup 1) were collected and stored at −20 °C till further use. The separated sperm pellets were subsequently washed six times with PBS as described above and supernatants from each washing (i.e., Sup 2 to Sup 7) were collected. Finally, supernatant 1, 4 and 7 were included in the study. The genomic DNA was isolated from the sperm pellet using GeneipureID™ DNA isolation kit (mdi, India).

The quantitative real-time PCR (qPCR) was conducted for the detection of reporter gene using the wash supernatants and DNA isolated from the final pellet of sperm cells. Specific primer pairs for enhanced green fluorescent protein (EGFP) and the reference gene, β-actin were designed using Primer Express (Ver 3.0). The following primers were used: EGFP 5′-CCGACCACTACCAGCAGAACAC-3′, 5′-CTCGTTGGGGTCTT TGCTCAG-3′; β-actin 5′-AGCTCGCCATGGATGATGA-3′, 5′-TGCCGGAG CCGTTGT-3′. All PCR reactions were performed in triplicates in a reaction volume of 20 μl containing 1× Power SYBR® Green Master Mix