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Testis mediated gene transfer: In vitro transfection in goat testis by electroporation

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ABSTRACT

Testis mediated gene transfer (TMGT) is a potential tool for making transgenic mice having more than 90% success rate. However, this method needs further standardization before it can be adapted in other species including livestock. In order to standardize the TMGT in goat, buck testes (n = 20) collected from the slaughter house were injected with a vector driving green fluorescent protein (GFP) expression under a cytomegalovirus (CMV) promoter. Then, the testes were subjected to electroporation with predetermined voltage, pulse length, pulse interval and number of pulses. Seminiferous tubules were isolated from the electroporated testis and cultured in-vitro. The expression was checked at regular intervals. Green fluorescence was observed on different days in different samples. It suggests transient integration of the plasmid into the seminiferous tubules. This in-vitro transfection of seminiferous tubule using electroporation will provide valuable baseline information.

1. Introduction

Transgenic animals have become valuable tools for both basic and applied research. There are four main methods of producing transgenic animals viz., DNA microinjection (Gordon et al., 1980), retroviral mediated gene transfer (Robertson et al., 1986), embryonic stem cell mediated gene transfer (Torres, 1998) and nuclear transfer (Wilmut et al., 1997). Among them the most widely used one is the DNA microinjection method (Gordon et al., 1980).

However, it is inefficient with a success rate of 1–4%, results in random integration into the host genome and often show variable expression due to position effects (Pursel and Rexroad, 1993; Wall, 1996). Although the retroviral mediated transgenic animal production has nearly 100% efficiency, it is often difficult to transfer a transgene of a larger size (Hu and Pathak, 2000). Embryonic stem cell mediated gene transfer and nuclear transfer require high skill and costly resources. Use of a sperm cell as a vehicle for the introduction of exogenous DNA into an oocyte at the time of fertilization seems to be a simple and obvious alternative. Brackett et al. (1971) demonstrated for the first time the ability of mammalian spermatozoa to take up and transfer exogenous DNA. Lavitrano et al. (1989) used sperm cells that were incubated with the transgene for in vitro fertilization. This sperm mediated gene transfer (SMGT) method though is very simple and convenient, compared with the more established pronuclear microinjection method, but could not be replicated in several species including chicken, cow and fish (Brinster et al., 1989). In order to circumvent these limitations several experiments have been carried out by different researchers. All these experiments fall into two distinct categories: auto and augmented uptake. The auto uptake as the name indicates is based on the intrinsic ability of the sperm cells to capture and transfer exogenous DNA, when DNA is incubated with sperm cells in vitro. But with this approach, most of the experiments failed to produce transgenic animals (Smith, 1999). In order to augment the uptake of exogenous DNA, sperm cells were in vitro treated with chemicals (e.g. liposomes, calcium-phosphate precipitate) or subjected to the physical method (e.g. electroporation). Chemical methods mediated transgenesis have a low success rate such as 9.5% for calcium-phosphate-precipitate (Sato et al., 1994), 0% for liposome mediated (Bachiller et al., 1991) and 20% for linker based methods (Epperly, 2007). The physical methods which primarily include electroporation had been mainly used to test the ability of spermatozoa to carry foreign DNA into the bovine oocyte rather than the production of transgenic animals (Gagne et al., 1991).

Since the results obtained by sperm mediated gene transfer are very unpredictable and also have low reproducibility (Brinster et al., 1989; Smith, 1999), an alternative approach of in vivo transfection of sperm cells referred as testis mediated gene transfer (TMGT) has been proposed. The ultimate aim of TMGT approach is to produce transgenic spermatozoa through transfection of undifferentiated spermatogonial stem cells (Dhup and Majumdar, 2008). In this study, in vitro gene transfer to the testis by DNA injection into seminiferous tubules and subsequent electroporation (EP) was performed using enhanced green fluorescent protein (EGFP) as a marker. Since most of the studies were reported only in laboratory animals we tried to find out whether the

Abbreviations: TMGT, testis mediated gene transfer; GFP, green fluorescent protein; CMV, cytomegalovirus; DMEM, Dulbecco's Minimum Essential Media; IRES, internal ribosome entry site; ECMV, encephalomyocarditis virus; EGFP, enhanced green fluorescent protein.

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Table 1
Summary of observations made on different days.

Plasmid concentration (μg/μl)	Wells showing fluorescence on different days															Total	Experiment no.
	1st (after 24 h)	3rd	5th	7th	9th	11th	13th	15th	17th	19th	21st	23rd	25th	27th	29th		
0.1	A1	A1	C4				A5, B2		C2							6	1
0.3	C2, D1				A2			A4								4	
0.5		A5										C4				2	
1.0										D3						1	
0.1	C5	C3			D1	D1	A3									5	2
0.3	C6	C6					B1									3	
0.5	A4		B3						B5							3	
1.0	B5	B5														2	
0.1	A2, C4C5	A2, C5		D5	D5											7	3
0.3	A5		B3	B3	B3											4	
0.5		C2, B6	C2			B5	B5									5	
1.0	B2			D4	D4				A3							4	

foreign gene will be incorporated into the germ cells of large animals or not.

2. Material and methods

2.1. Plasmid DNA

The eukaryotic GFP expression vector p*IRES2-EGFP* (Clontech, USA) was obtained from the National Institute of Immunology, New Delhi, India. 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).

2.2. In vitro electroporation of caprine testis

The testes of approximately 6–8 month old bucks (n = 20) collected from local abattoir, Bareilly, Uttar Pradesh, were used in this study. Immediately after collection, the testis was transported to a laboratory in PBS on ice. In the laboratory, the testis was washed with PBS containing 50 μg/ml Gentamicin (Sigma, USA). Extra fat surrounding the testis was removed using sterile scissors. Approximately 1 ml of injection solution containing four different concentration levels of plasmid (0.1, 0.3, 0.5, 1.0 μg/μl) was injected slowly into the interstitial space at eight different sites of the testis using a 1 ml syringe. Electric pulses were delivered with an electric pulse generator (Electrosquare Porator, ECM830, BTX, USA). The testis was held between a pair of tweezer-type electrodes and square electric pulses were applied four times and again four times in the reverse direction for 50 ms duration. We used electric pulses of 50 V and an inter-pulse interval of 1 s.

2.3. Culture of seminiferous tubules

Immediately after electroporation, for the isolation of seminiferous tubules, the testis was excised and decapsulated by making a small incision in the tunica albuginea and gently expressing the contents in to an aseptic surface. The decapsulated testis was kept in PBS containing 50 μg/ml Gentamicin (Sigma, USA). The seminiferous tubules were isolated mechanically. Briefly, the testis was cut into different pieces using a sterile surgical blade. The pieces of interstitial tissue were minced with scissors and the seminiferous tubules were isolated by stripping off the adjacent connective tissue using forceps and transferred to a separate Petri plate containing culture medium. The culture medium used was Dulbecco's Minimum Essential Media (DMEM) and Ham's F-12 (Gibco, USA) in 1:1 ratio containing 10% FBS and antibiotic Gentamicin @ 20 μg/ml. The isolated seminiferous tubules were transferred to a 24 well plate containing the culture medium and the plate was incubated in CO_2 incubator at 37 °C, 100% RH and 5% CO_2. The culture was maintained for up to four weeks and the medium was changed every 24 h. In order to rule out the possibility of autofluorescence in the tissue, a naïve control was set-up in which the testis was injected with autoclaved distilled water and electroporation was skipped. Further, to ascertain the effect of electroporation, an electroporation control was also set-up. In this control, the testis was injected with a known concentration of plasmid (0.5 μg/ml) but electroporation was not carried out.

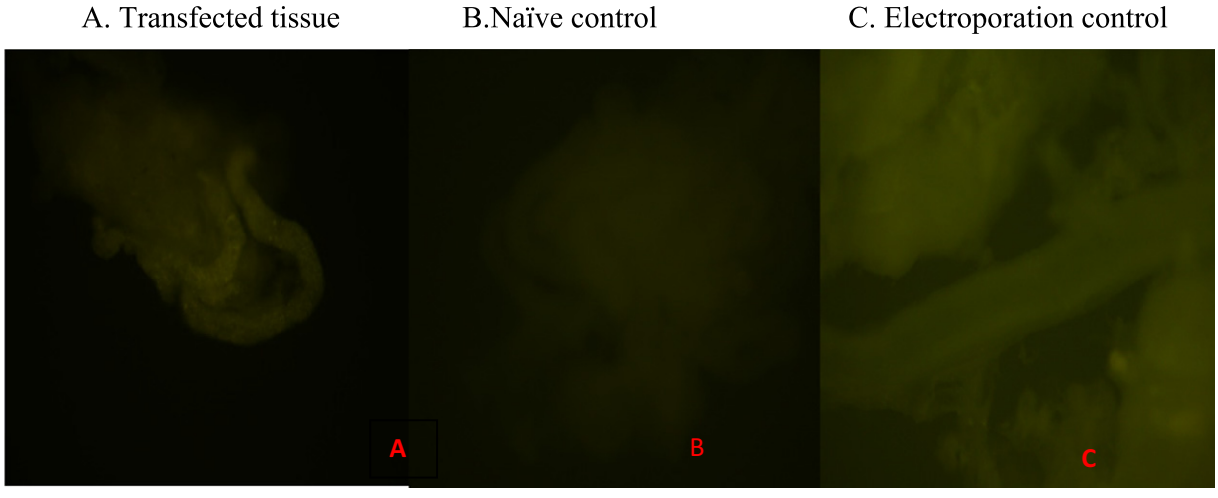

Fig. 1. Comparison between transfected tissue and negative tissue (both naïve control and electroporation control) (4×). A. Transfected tissue. B. Naïve control. C. Electroporation control.