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Expression of fluorescent reporter protein was not obtained in ovine embryos produced through *in vitro* fertilization-sperm mediated gene transfer (IVF-SMGT)

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ABSTRACT

Sperm mediated gene transfer (SMGT) has been reported to be a powerful tool for producing transgenic animals on a mass scale using spermatozoa as vectors for exogenous DNA. In this study the possibility of using *in vitro* fertilization (IVF) –SMGT to produce transgenic ovine embryos was investigated for the first time. For this purpose, sperm were obtained from the epididymal testicle areas of 4 rams and different concentration of DNA (0.4, 0.8 and 1.6 μg) and TurboFect (0.25, 0.5, 1 and 2 μg) were used for sperm transfection (1×10^6). Enhanced-GFP-expressing vector pEGFP-N1 was used as the carrier. In order to evaluate the performance of transgenic sperm, *in vitro* fertilization technology was used. After the preparing oocytes received from the ovaries of slaughterhouse origin, oocytes with more than three layers of granulosa and uniform cytoplasm were selected and matured in TCM-199 medium containing 10% fetal calf serum, follicle stimulating hormone (FSH) (5 μg/ml), β-17 estradiol (1 μg/ml) and sodium pyruvate 0.81 (Mm). Bracket and Oliphant's (BO) medium and modified Charles Rosenkrans medium with amino acids (mCR2aa) were used for *in vitro* fertilization and culture, respectively. Results showed that transfected sperm with different concentrations of DNA and TurboFect carrier were unable to transfer the GFP gene to *in vitro* matured oocytes as the GFP gene was not expressed in neither zygotes nor morula stage embryos. Further optimization for SMGT improvement such as different transfection reagent and method like electroporation, using antioxidants in transfection medium to overcome the apoptosis and also separation of transfected sperm from untransfected ones before insemination have been suggested.

1. Introduction

Transgenic livestock production is of particular interest in agriculture and biomedicine (Zaniboni et al., 2016). The first transgenic livestock was produced in 1985 by microinjection of foreign DNA into zygotic pronuclei. This was the method of choice for more than 20 years (Kues and Niemann, 2011), and transgenic mice, sheep, pigs, and cattle have been successfully produced using this technique (Garcia-Vazquez et al., 2009), but besides being expensive, this technique is still inefficient when used to generate transgenic farm animals (0.5–4%) (Garcia-Vazquez et al., 2011). More efficient protocols are now available (Kues and Niemann, 2011), including virus-mediated transgenesis, somatic cell nuclear transfer (SCNT) and sperm mediated gene transfer (SMGT) (Zaniboni et al., 2016). These methods vary in efficacy and safety. Technical and safety considerations limit the use of viral methods, especially in agricultural applications. In addition, the efficiency of non-viral methods, compared to viral methods is very low (Katebi et al., 2016).

Since 1971 there is evidence that a "heterologous genome" can be incorporated into a mammalian spermatozoon. Twenty years later it was shown that circular DNA can be incorporated into mouse epididymal spermatozoa by simple incubation and can be transmitted to oocytes resulting in the creation of transgenic animals (Hoelker et al., 2007). SMGT is based on the ability of sperm cells to bind, internalize and transport exogenous DNA into an oocyte during the process of fertilization, so uses sperm as a natural vector to transfer transgenes (Lavitrano et al., 1989; Kang et al., 2008; Garcia-Vazquez et al., 2011). However, the theory has not been well documented in different laboratories across the globe (Eghbalsaied et al., 2015).

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The SMGT method, if proved to be reproducible, would be the simplest, cost-effective, most rapid and mass gene transfer method to produce transgenic farm animals (Hoelker et al., 2007; Feitosa et al., 2010; Katebi et al., 2016). The SMGT strategy with the use of sex-sorted semen can also be an attractive approach to the generation of genetically-transformed male and female embryos/progeny derived from *in vitro* or *in vivo* fertilization (Zaniboni et al., 2016).

Although previous studies have resulted in the creation of transgenic animals in several species, including some invertebrate and insects, amphibians, fish, chicken, mouse, pigs and cattle, effectiveness remained low (Hoelker et al., 2007; Lanes et al., 2009; Bacci et al., 2009). Several factors determine the success of SMGT including the donors of spermatozoa, incubation media, exogenous DNA size and type and the assisted reproductive technique used (Garcia-Vazquez et al., 2011). DNA binding efficiency varies widely ranging from 0.3 to 78% among the sperm of different species. Consequently, the success rate of SMGT also varies in different animal species (Pramod et al., 2016). With respect to SMGT effectiveness both species specific effects and variability within individuals of one species were reported (Hoelker et al., 2007).

In order to improve the DNA uptake efficiency of sperm cells, several strategies have been employed, including treatment of spermatozoa with Triton-X (Hoelker et al., 2007), the use of chemical transfection reagents like liposomes (Bachiller et al., 1991) or DMSO (Shen et al., 2006), electroporation, monoclonal antibodies to link foreign DNA to the spermatozoa surface (Pramod et al., 2016) and restriction enzyme- mediated integration (REMI) (Camposa et al., 2011).

SMGT without intracytoplasmic sperm injection (ICSI) have not been reproduced to our knowledge in sheep. The aim of the current study was to examine the possibility of generating transgenic ovine embryo using sperm vector with the assistance of TurboFect transfection reagent.

2. Material and methods

2.1. Chemicals

All the culture media, growth factors, fetal bovine serum (FBS) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and the plasticware was purchased from Falcon (Paignton, UK) unless stated otherwise.

2.2. Plasmid

The enhanced green fluorescent protein (EGFP) plasmid construction (pEGFPN1, 5.4 kb) used for our experiments contained the CMV promoter and the enhanced GFP gene. Plasmids were extracted using Plasmid Mega Kit (Qiagen) and the StuI restriction enzyme (Takara, Japan) was used to prepare linearized plasmid following the manufacturer's instructions and digestion efficiency was checked by 1% agarose gel electrophoresis.

2.3. Transfection of fibroblast cells

The ovine fibroblast cell line were established from sheep ear skin as described by Shah et al. (2008). One day before transfection, 0.5×10^5 of cells plated in 300 μl of growth medium (DMEM, 10% FBA and 50 μg/ml gentamicin) so that cells were 75% confluent at the time of transfection. In order to evaluation of vector, different concentration of DNA (0.1, 0.2, 0.4 and 0.8 μg) and TurboFect (Thermo Scientific/R0531) (0.5, 1 and 2 μg) were diluted in 50 μl of transfection medium separately, and incubated for 5 min at room temperature. Then, the diluted DNA was added to diluted TurboFect (total volume = 100 μl) and incubated for 20 min at room temperature. 100 μl of complexes was added to each well containing cells and mixed gently by rocking the plate back and forth. The cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h prior to testing for transgene expression by fluorescence microscopy.

2.4. Collection of epididymal sperm

Four ram testes collected from a nearby abattoir slaughterhouse immediately after slaughter and were brought to the laboratory in an ice chest. In the laboratory, the testes were rinsed twice with normal saline and were then trimmed to remove the extra testicular tissue and washed properly with saline containing 0.1% streptomycin sulphate. Connective tissue covering the cauda epididymis was removed by careful dissection, with care to avoid rupturing blood vessels or the epididymal duct. Epididymal sperm collected as described by Garde et al. (1994). In summary, sperm was recovered from the cauda of epididymis by injecting PBS retrogradely through the ductus deferens. Collected sperm was washed once by centrifugation with PBS medium at 600*g* for 7 min. The pellet obtained was suspended in BO media and evaluated for motility.

2.5. Transfection of sperm

In order to transfection of sperm, different concentration of DNA (0.4, 0.8 and 1.6 μg) and TurboFect (0.25, 0.5, 1 and 2 μg) were diluted in 25 μl of transfection medium separately, and incubated for 5 min at room temperature. Then, the diluted DNA was added to diluted TurboFect (total volume = 50 μl) and incubated for 20 min at room temperature. 1×10^6 sperm were added to 50 μl of DNA-TurboFect complexes and mixed gently by rocking the plate back and forth. The cells were then incubated at 37 °C in a CO_2 incubator (5% CO_2 in air) for 20 min and added to 50 μl of BO media containing matured ovine oocytes for *in vitro* fertilization.

2.6. In vitro *fertilization*

Sheep ovaries collected from a nearby abattoir were brought over to the laboratory in phosphate-buffered saline (PBS) containing 100 IU/ml penicillin and 50 mg/ml streptomycin at 30–34 °C within 3 h of slaughter. Ovarian follicles of 2–8 mm diameter were aspirated using an 20-gauge needle attached to a 10-ml syringe to obtain cumulus-oocyte complexes (COCs). Those having more than 3 layers of compact unexpanded cumulus cells and homogenous evenly-granular ooplasm were transferred to 100-μl droplets (15–20 COCs per droplet) of *in vitro* maturation (IVM) medium, which consisted of TCM-199 containing 10% FBS, 10% buffalo follicular fluid, 5 mg/ml porcine FSH, 1 mg/ml oestradiol- 17β, 0.81 mM sodium pyruvate and 50 mg/ml gentamicin sulfate, and were cultured under mineral oil in a petri dish in a CO_2 incubator (5% CO_2 in air) at 38.5 °C for 24 h. For *in vitro* fertilisation the oocytes were washed twice with BO medium and were transferred to 50-μL droplets (15–20 oocytes per droplet) of capacitation and fertilisation BO medium (washing BO medium containing 10 mg/ml fatty acid-free bovine serum albumin (BSA)). The spermatozoa were prepared for fertilisation as per the protocol established by Chauhan et al. (1997). Oocytes were then inseminated using a final concentration of 1×10^6 motile spermatozoa ml^{-1} and incubated under mineral oil in a CO_2 incubator (5% CO_2 in air) at 38.5 °C for 18 h. The cumulus cells were then removed from the presumed zygotes, after which these were cultured in mCR2aa containing 0.6% BSA for 48 h in groups of 10 per droplet. Following this, the cleaved embryos obtained were cultured in the *in vitro* culture (IVC) medium (mCR2aa + 0.6% BSA + 10% FBS) for up to 4 days until morula were obtained, with a change of medium every 48 h.