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Improved expression of green fluorescent protein in cattle embryos produced by ICSI-mediated gene transfer with spermatozoa treated with streptolysin-O

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ABSTRACT

The ICSI-sperm mediated gene transfer (ICSI-SMGT) has been used to produce transgenic mice with high efficiency; however, the efficiency of this technique in farm animals is still less than desirable. Pretreatment of sperm with membrane destabilizing agents can improve the efficiency of ICSI in cattle. The objective of the present study was to evaluate streptolysin-O (SLO) as a novel treatment to permeabilize the bovine sperm membrane and assess its effect on efficiency of generating transgenic embryos by ICSI-SMGT. First, there was evaluation of the plasma membrane integrity (SYBR/PI), acrosome membrane integrity (PNA/FITC), DNA damage (TUNEL) and binding capacity of exogenous DNA (Nick Translation) in bull sperm treated with SLO. Subsequently, there was assessment of embryonic development and the efficiency in generating transgenic embryos with enhanced expression of the gene for green fluorescent protein (EGFP). Results indicate that SLO efficiently permeabilizes the plasma and acrosome membranes of bull spermatozoa and increases binding of exogenous DNA mostly to the post-acrosomal region and tail without greatly affecting the integrity of the DNA. Furthermore, treatment of bull spermatozoa with SLO prior to the injection of oocytes by ICSI-SMGT significantly increased the rate of embryo expression of the EGFP gene. Future experiments are still needed to determine the effect of this treatment on the development and transgene expression in fetuses and animals produced by ICSI-SMGT.

1. Introduction

Transgenesis is a very useful biotechnological approach with diverse applications in the pharmaceutical industry, human

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medicine and even the agricultural sector (Lavitrano et al., 2013).

Nevertheless, producing transgenic cattle is difficult and costly due, in part, to the low efficiency of transgenesis techniques and the long period of time required to assess the transgene and produce the desired recombinant protein (Monzani et al., 2016).

Different methods have been considered for production of transgenic cattle, including pronuclear microinjection (Loskutoff et al., 1986), lentiviral vectors (Hofmann et al., 2004), somatic cell nuclear transfer (SCNT) (Wang et al., 2015), embryonic stem cells (Malaver-Ortega et al., 2012) and more recently the CRISPR/Cas9 system (Heo et al., 2015). Currently, one of the most used methods to produce transgenic cattle is SCNT, due to the relatively greater rates of transgenic embryo generation (Monzani et al., 2016). The subsequent viability of the clones, however, limits the overall efficiency in transgenesis (Cibelli et al., 2002).

One method proposed several years ago to be simple, economical and rapid for large-scale transgenesis in farm animals is sperm-mediated gene transfer (SMGT). This technique is based on the ability of spermatozoa to bind, internalize and transport exogenous DNA molecules within the oocyte during fertilization to enable the generation of transgenic embryos and/or offspring (Lavitrano et al., 1989; Lavitrano et al., 2013). First transgenic mice generated by SMGT were reported in 1989 (Lavitrano et al., 1989). Later, the same authors reported that SMGT could be used to produce transgenic farm animals, establishing transgenic pigs lines capable of efficiently expressing the gene for human decay-accelerating factor (Lavitrano et al., 2002). Despite these promising findings, efficiency of this technique continues to be less than desirable, in part to lack of efficient binding and integration of exogenous DNA in the sperm nucleus, which prevents a larger number of transfected spermatozoa from fertilizing the oocytes (Alderson et al., 2006; Anzar and Buhr, 2006; Eghbalsaied et al., 2013).

Transfection methods such as lipofection, electroporation, protamine sulfate and restriction enzyme-mediated integration (REMI) have been addressed as a strategy to increase the rate of exogenous DNA uptake in bull spermatozoa, the results however, continue to be inconsistent, irreproducible and in many cases they reduce the quality of sperm (Alderson et al., 2006; Eghbalsaied et al., 2013; Cavalcanti et al., 2016; Arias et al., 2017).

Perry et al. (1999) suggested combining intracytoplasmic sperm injection (ICSI) and SMGT to destabilize the sperm membrane with chemical or physical treatments prior to sperm injection (Perry et al., 1999). The use of membrane destabilizing agents, however, is thought to damage the sperm DNA, thus reducing the embryonic developmental potential (Yanagimachi, 2005; Hoseini Pajooh et al., 2016; Canel et al., 2018). This is why recent studies have focused on developing less invasive transfection methods for sperm cells (Moisyadi et al., 2009; Sim et al., 2013; Sanchez-Villalba et al., 2018). Sim et al. (2013) demonstrated that ICSI-SMGT efficiency in mice can be improved when the sperm membrane is permeabilized with streptolysin-O (SLO). SLO forms stable pores in the sperm membrane and allows for exogenous DNA uptake without greatly affecting the sperm nuclear DNA, a problem that occurs with other compounds (Johnson et al., 1999). The objectives of the present study, therefore, were to establish cattle sperm incubation conditions to permeabilize the plasma and acrosome membranes using SLO and assess the effects of SLO treatment on embryonic development and the generation of transgenic embryos by ICSI-SMGT.

2. Materials and methods

Unless otherwise indicated, all the reagents used were acquired from Sigma-Aldrich (St Louis, MO, USA).

2.1. Exogenous DNA and labeling

The plasmid used was pCX-EGFP, kindly supplied by Dr. Masaru Okabe of the University of Osaka, Osaka, Japan. The plasmid was labeled with fluorescein isothiocyanate (FlTC)-12-dUTP (Thermo Fisher Scientific, Inc., MA, USA) using the Nick Translation System (Thermo Fisher Scientific, Inc., MA, USA) according to the manufacturer's instructions to identify the presence and location of the plasmid in spermatozoa with flow cytometry and confocal microscopy, respectively.

2.2. Preparation of spermatozoa for incubation with exogenous DNA

Cryopreserved semen from a commercial bull with proven fertility both *in vivo* and *in vitro* was used (Alta Genetics Inc., Alberta, Canada). The frozen sperm were thawed in a water bath for 1 min at 38.5 °C and selected by Percoll gradient (Parrish et al., 1995). Selected cryopreserved spermatozoa were washed in Ca^{2+} and Mg^{2+} free Hank´s balanced salt solution (HBSS) and incubated with 5, 10 and 20 U/ml SLO. A control group without treatment was also included. Subsequently, 500 ng of exogenous DNA was added to 1×10^6 sperm and incubated for 30 min at 37 °C.

2.3. Evaluation of exogenous DNA binding in spermatozoa

To assess the capacity of exogenous DNA to bind to spermatozoa and the effect of this treatment on cell viability, the sperm subjected to each treatment were incubated with DNA marked with fluorescein isothiocyanate (DNA-FITC) and propidium iodide (PI) for 30 min and immediately evaluated by flow cytometry (FACS CANTO II, Becton Dickinson, Mountain View, USA). Later, the fluorescence signal was used to determine the amount of exogenous DNA bound to spermatozoa.

2.4. Detection of exogenous DNA

Spermatozoa subjected to each treatment were incubated with FITC-labeled DNA and evaluated by confocal microscopy