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Prolonged Expression of Exogenous GFP Gene in the Porcine Embryos generated by Intracytoplasmic Sperm Injection-Mediated Gene Transfer

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ABSTRACT

Understanding the behavior of transgenes introduced into oocyte or embryos is essential for evaluating the methodologies for transgenic animal production. To date, many studies have reported the production of transgenic pig embryos with, however, low efficiency in environment of blastocyst production. The aim of present study was to determine the expression and duration of transgene transferred by intracytoplasmic sperm injection-mediated gene transfer (ICSI-MGT). Embryos obtained from the ICSI-MGT procedure were analysed for the expression of GFP and then for the transmission of the transgene. Briefly, fresh spermatozoa were bound to exogenous DNA after treatment by Triton X-100 and Lipofectin. When ICSI-MGT was performed using sperm heads with tails removed, the yield of blastocyst (25.3%), treated with Lipofectin (18.8%) and Triton X-100 (19.2%) were observed. Treatments of Lipofectin or Triton X-100 did not further improve the rates of blastocysts. Moreover, the apoptosis rates of embryos were obtained from the control and LIpofectin groups (8.7%, 9.7%, respectively), but were significantly higher in the Triton X-100 group (13.0%). Our results demonstrated that ICSI-MGT caused minimal damage to oocytes that could develop to full term. Moreover, the embryos derived by ICSI-MGT have shown prolonged exogenous DNA expression during preimplantation stage *in vivo*. However, more efforts will be required to improve the procedures of both sperm treatments cause of high frequency of mosaicisms.

(Key words : transgenic pig, ICSI-MGT, GFP expression)

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a valuable assisted reproduction technology for studying fertilization, human infertility and producing transgenic animals. Their value for research is enhanced by the physiological and anatomical similarities between pig and human (Lunney, 2007). In 1989, Lavitrano *et al.* (1989) firstly demonstrated that transgenic animal could be produced by *in vitro* fertilization combined with sperm-mediated gene transfer (SMGT) technique based on the concept of using sperm as a vector for introducing exogenous DNA. After a decade, in 1999, Perry *et al.* (1999) produced transgenic mice by ICSI using sperm. This technique was named as ICSI-mediated gene transfer (ICSI-MGT) and was a powerful technique for the generation of transgenic mice. Some of the beneficial effects of ICSI-MGT were that it can avoid low transgenic efficiencies compared to pronuclear microinjection (Eyestone, 1999) and that it had lower rates of imprinting problems than somatic cell nuclear transfer (Rideout *et al.*, 2001). Sperm-mediated gene transfer (SMGT) is based on the ability of sperm to bind, internalize, and transport exogenous DNA into an oocyte during fertilization (Lavitrano, 1989; 1992; 1997; Francolini, 1993). In particular, SMGT harnesses the ability of the spermatozoa bind and exogenous DNA before the removal of the seminal plasma and to then transfer it to the oocyte when fertilization occurs. This transgenesis method can potentially be applied to all animal species with a gamic reproduction (Bacci, 2007). However, for successful application of ICSI-MGT in domestic species, especially in pigs, it is important to improve the binding ability of sperm with exogenous DNA. Therefore, in this study, the effect of sperm treatments for DNA binding before performing ICSI-MGT was examined. Primarily, the binding ability and the expression patterns of the exogenous EGFP gene were investigated for long term. Then, the transgene expression and blastocyst

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production rates and quality were determined.

MATERIALS AND METHODS

All animal in the present study were used in accordance with the guidelines of the National Institute of Animal Science, Rural Development Administration, Suwon, South Korea, and was approved by the committee at National Institute of Animal Science. Unless stated otherwise, all chemicals and reagents used present study were purchased from Sigma-Aldrich Chemicals.

1. *In Vitro* Maturation of Oocytes

Ovaries were obtained from a local abattoir and cumulus oocyte complexes (COCs) were aspirated from follicles (3 to 6 mm of diameter). Around 50 COCs were matured in 500 μl of tissue culture medium (TCM)-199 (Gibco-BRL) containing PVA (0.1%), D-glucose (3.05 mM), sodium pyruvate (0.91 mM), penicillin G (75 μg/ml), streptomycin (50 μg/ml), cysteine (0.57 mM), LH (0.5 μg/ml), FSH (0.5 μg/ml) and EGF (10 μg/ml) for 42～44 h at 38.5℃ in an atmosphere of 5% CO_2 and 95% air.

2. Sperm Collection and Treatments

Freshly collected boar semen by globed hand method was washed twice by centrifugation at 800 g for 5 min in D-PBS (Gibco-BRL) supplemented with 0.1% BSA. Firstly for treatment of Triton X-100, suspended spermatozoa (concentration of 1 to 2×10^6 sperms/ml) were washed twice in nuclear isolation medium (NIM) consisting 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 3.0 mM EDTA (pH 7.2). Triton X-100 to a final concentration of 0.05% (v/v) was added in the sperm suspension and reacted for 60 s. The reacted spermatozoa with Triton X-100 were centrifuged twice for 1 min at 20,000 g at 2℃ and re-suspended in ice-cold NIM. The 200～400 μl NIM including spermatozoa was mixed with 10 ng/μl of DNA (7.4 kb sized pCX-EGFP/Neo) which was linearized by Sal I restriction enzyme. Secondary for transfection of spermatozoa, 1.5 ml of D-PBS containing 0.5 μl of Lipofectin (Gibco-BRL) and 10 ng/μl of DNA (7.4 kb sized pCX-EGFP/Neo) were incubated at 17℃ for 1 h. Then, the suspended spermatozoa (concentrations of 1 to 2×10^6 sperms/ml) were incubated in the DNA-Lipofectin mixture for 6 h before ISCI.

3. Intracytoplasmic Sperm Injection and Oocyte Activation

Cumulus cells were denuded by gentle pipetting in TCM-199 containing 0.1% hyaluronidase. Oocytes with visible first polar body and morphologically normal were conducted for the ICSI. After ICSI, the oocytes were activated chemically. Briefly, the oocytes were treated with 2 mM of A23187 for 5 min and 7.5 μg/ml of cytochalasin B for 6 h. Around 30～40 oocytes per 500 μl of NCSU-23 medium were cultured at 39℃ in 5% CO_2.

4. Apoptosis Analysis and Cell Counting

Fully expanded blastocysts were fixed in PBS containing 3.7% paraformaldehyde for 1 h at room temperature. The blastocysts were permeabilized in PBS containing 0.5% Triton X-100 for 30 min. The blastocysts were treated by TUNEL reaction mixture (TMR red; Roche Diagnostics) for 1 h at 39℃ in a dark place. Then, the blastocysts were counterstained by 2 μg/ml of DAPI (Roche Diagnostics) for 15 min, and mounted onto slides with anti-fading gel mount (Molecular Probes). The blastocysts were examined for the numbers of apoptotic nuclei and total numbers of nuclei using a fluorescent microscope (IX-71; Olympus). Apoptosis ratio was calculated by total cells per apoptotic cells.

5. Embryo Transfer and Recovery

Estrus cycles of gilts were synchronized by feeding with Regu-mate (Hoechst Roussel Pharmaceuticals Inc.). Briefly, the recipients were fed Regu-mate (20 mg/day) from Day 1 to 9 of estrus cycle (Day 0 being the day of onset of the estrus), and intramuscularly injected with 750 IU hCG on 5 days after the final Regu-mate feeding to induce ovulation. Two days after hCG injection, embryos were transferred into the oviduct of recipients. On day 5, 7 and 15 after embryo transfer, embryos were recovered by flushing from uterine horns using PBS containing 0.1% BSA. Developmental competence of recovered embryos and expressions of GFP were observed under the fluorescence microscope (Olympus).

6. Statistics

The statistical significance among treatment groups in each experiment was determined by using General Linear Models Procedure and *t*-test of SAS.

RESULTS AND DISCUSSION

Based on preliminary results, the embryos activated chemically by A23187 (36.0%) showed higher blastocysts formation

rates than electrical activation group (28.9%) significantly ($p<0.05$). Therefore, all embryos after ICSI were activated by chemically. In Table 1, the cleavage rates were not significantly different in groups. However, the yields of blastocysts were significantly ($p<0.05$) reduced when the sperm treated with Lipofectin (18.8%) and Triton X-100 (19.2%) compared to non-treated control group (25.3%). These result suggested that the Lipofectin and Triton X-100 treatments was affected the embryonic development *in vitro*. However, the apoptotic cells were significantly ($p<0.05$) higher only in the group of Triton X-100 (13%) compared to groups of non-treated (8.7%) and Lipofectin (9.7%) with no significant difference in total cell numbers (data not shown). These results showed similarity to previous report by Bachiller *et al.* (1991). We could confirm the possibility of liposomes-mediated exogenous DNA uptake by sperm cells. Although, on the other hand, treatment by Triton X-100 firstly reported in mice (Ahmadi and Ng, 1997; Kasai *et al.*, 1999) did not affect beneficially in embryonic development but it has significant effect on the membrane-damaged sperm in human (Ahmadi and Ng, 1999). These results suggested that membrane-damage sperm can effectively enhanced the success rate of ICSI in mice and human. Our result of membrane-damaged sperm by Triton X-100 also confirmed that the porcine sperm membrane has similarity with human sperm membrane as previous report by Lee *et al.* (2004). As shown in Table 2, the rates of GFP expression were observed. Briefly, the Lipofectin group (4.2%) showed significantly higher ($p<0.05$) expression of whole blastocysts compared to Triton X-100 group (0.9%) during a total expression rates showing no significance (36.4% and 40.6% in Lipofectin and Triton X-100 groups respectively). These result suggested that the Lipofectin treatment was less harmful than Triton X-100 treatment regarding only whole expression of blastocyst. Additionally, a total 295 ICSI-MGT-derived embryos were transferred to recipients and 107 embryos were recovered on 5, 7 and 15 days. The GFP expression rates in each recovery day were 26.9%, 34.4% and 43.4% on day 5, 7 and 15 respectively. The expression of GFP in elongated embryos (recovered on day 15) was prolonged during *in vitro* culture for 7 days (Fig. 1).

In summary, taken together, the results demonstrated that ICSI-MGT caused minimal damage to oocytes which could develop to full term *in vitro* and *in vivo* state. Moreover, the embryos derived by ICSI-MGT have shown prolonged exogenous DNA expression during preimplantation stage *in vivo*.

Table 1. *In vitro* development of porcine embryos after ICSI-MGT

Sperm treatments	ICSI	Cleaveage (%)*	Blastocysts		
			Yield (%)*	Cell numbers	Apoptosis ratio (%)*
None-treated	75	62.7	25.3[a]	36.3	8.7[a]
Lipofectin	96	59.4	18.8[b]	31.4	9.7[a]
Triton X-100	116	62.1	19.2[b]	36.8	13.0[b]

* Percentages are expressed as the mean value of 5 replicates in each group.
[a,b] Different superscripts denote significant differences within columns ($P<0.05$).

Table 2. GFP expression patterns during *in vitro* culture of porcine embryos after ICSI-MGT

Sperm treatments	No. of oocytes injected	Expression patterns	No. of (%) GFP expressing embryos*			
			1-cell	>2-cell	Blastocyst	Total
Lipofectin	96	Whole	3 (3.1)[a]	4 (4.2)[a]	4 (4.2)[a]	11 (13.5)[a]
		Mosaic	0 (0.0)[b]	17 (17.7)[b]	5 (5.2)[a]	22 (22.9)[b]
Triton X-100	116	Whole	7 (6.0)[a]	9 (7.8)[a]	1 (0.9)[b]	17 (14.7)[a]
		Mosaic	5 (4.3)[a]	21 (18.1)[b]	4 (3.4)[a]	30 (25.9)[b]

* Percentages are expressed as the mean value of 4 replicates in each group.
[a,b] Different superscripts denote significant differences within columns ($P<0.05$).

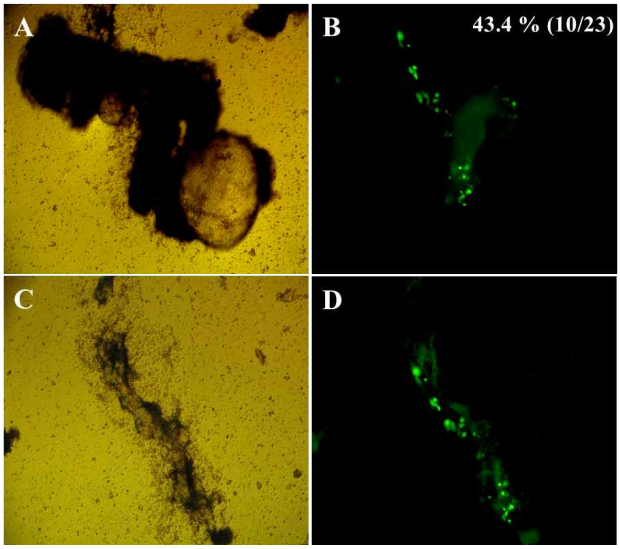

Fig. 1. Prolonged GFP expression of porcine preimplantation embryos. Elongated embryos were recovered at day 15 of embryo transfer after ICSI-MGT with sperm/DNA mixture. After recovery, elongated embryos were cultured for 1(A, B) or 7days(C, D) *in vitro*. Light microscope (A, C) and green fluorescence image (B, D) were observed on a fluorescence microscopy. GFP expression (green) in the porcine elongated embryos was prolonged during *in vitro* culture for 15 days. Magnifications: (A, B: x40 and C, D: x100).

Therefore, we thought that Triton X-100 and Lipofectin treatment was very useful to generate the sperm/DNA mixture for the ICSI-MGT and transgenic production. However, more efforts will be required to improve the procedures of both sperm treatments cause of high frequency of mosaicisms.

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