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Exogenous DNA Transfer by Intracytoplasmic Sperm Injection in Porcine Oocytes

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ABSTRACT

Sperm-mediated DNA transfer has a potential to markedly simplify techniques for the generation of transgenic animals. The exogenous DNA transfer by intracytoplasmic sperm injection (ICSI) procedure has been recently introduced in the production of transgenic animals. In this study, the developmental competence and the expression rates of transgene were investigated after injection of spermatozoon or sperm head with enhanced green fluorescent protein (EGFP) gene into the mature porcine oocytes. The porcine oocytes were injected with intact sperm, membrane-disrupted sperm or sperm head. After injection, embryos were cultured in NCSU23 medium up to the blastocyst stage, and the developmental competence and expression rates were studied. The developmental rate (67.0%) of sperm injection group was higher than that (59.7%) of sperm head injection group, and the rates of EGFP expression were also significantly different between sperm injection and sperm head injection groups (42.1 vs 20.0%) ($P<0.05$). In the porcine oocytes injected with sperm treated with different methods of membrane disruption, the removal of sperm membrane did not alter the developmental competence of embryos. The rate of blastocysts at 7 days after injection with intact and membrane disrupted sperm were 15.0 and 14.2%, respectively. The EGFP expression rates, 38.4% in embryos injected with frozen-thawed sperm was higher than that, 22.4% of embryos injected with the Triton X-100 treated sperm. Prior to injection, sperm were cultured in different EGFP gene concentrations from 0.01 to 1ng/μl. However, no significant difference in developmental rates of embryos among different concentrations of EGFP gene were observed. The highest expression rate of EGFP gene, 37.4% was obtained from the embryos injected with spermatozoa treated with 0.1 ng/μl EGFP gene. These results suggested that exogenous DNA could be attached to the membrane disrupted sperm, and that these sperm could be used as a vector carrying foreign DNA into embryos.

(Key words : Exogenous DNA, ICSI, EGFP, Porcine)

Ⅰ. INTRODUCTION

Progression of gene modification technologies suggested cows producing complex collections of human antibodies or pigs producing replacement tissues and organs for humans. This progress continues to be made in expanding of genetic modification technologies beyond pronuclear microinjection (Gordon et al., 1980). Greater control over

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outcome of integration can be achieved by using mouse embryonic stem (ES) cells transfected with constructs capable of targeted mutagenesis by homologous recombination (Evans et al., 1981). Limitation in the available strategies for modifying mammalian germ lines have fueled a search for alternative methods, including the use of recombinant retrovirus to infect oocytes or preimplantation embryos (Chan et al., 1998) and spermatozoa as vehicles for DNA delivery during *in vitro* fertilization (IVF) (Lavitrano et al., 1989).

Spontaneous ability of sperm cells to bind exogenous DNA molecules can be exploited by using spermatozoa as vectors for delivering foreign genetic information to eggs during fertilization (Sciamanna et al., 2000), and genetically transformed animals can be generated using this method with variable degrees of success (Lavitrano et al., 1989;Maione, B. et al, 1998).

Recently, the gene transfer by intracytoplasmic sperm injection (ICSI) procedure has been introduced to produce transgenic mice (Perry et al., 1999) and pigs (Lazzereschi et al., 2000). The expression of the transgene in offspring of 20% and transmission to the F_1 generation in 40% of positive piglets was reported. ICSI has also been employed using spermatozoa in which the external membrane damaged or removed spermatozoa has been shown to be able to deliver foreign DNA to embryos (.Lazzereschi et al., 2000)

Using restriction enzyme mediated integration (REMI) and lipofection produced transgenic sperm have been used to produce transgenic embryos and live offspring (Shemesh et al, 2000). However, the use of ICSI for the production of transgenic animals appears to have at least an advantage over the other methods with high integration frequency. Live spermatozoa are used as a vector for introducing recombinant DNA into the oocytes *in vitro*. The delivery of exogenous DNA into mammalian oocytes by ICSI, and the production of EGFP-expressing embryos and live births in mouse were reported. (Chan et al. 2000).

The birth of transgenic pigs in the present study suggests that sperm-mediated transfer of DNA via ICSI may be possible in swine. Therefore these experiments were improved new effective method to introduce foreign DNA into embryos using spermatozoa.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation of Oocytes

Porcine ovaries were obtained from the slaughterhouse and transported to the laboratory at 37℃ in saline. Cumulus oocyte complexes (COC) were aspirated with an 18-gauge needle into a disposable 10 ml syringe from follicles of 3 to 6 mm in diameter. The COC were washed three times in TL-HEPES medium (Prather et al., 1995). Groups of 20 COC were matured in 100 μl of TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 2.2 g/l sodium bicarbonate, 10% porcine follicular fluid, 0.6 mM cystein, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamycin sulfate, 0.5 μg/ml FSH (Follitropin V; Vetrepharm, Canada), and 1 μg/ml estradiol-17β (Sigma Co., USA) under paraffin oil at 39℃ for 48 hr in 5% CO_2 in air.

2. *In Vitro* Fertilization of Oocytes

After the completion of culture, the oocytes were treated with 0.1% hyaluronidase in TL-HEPES, washed three times with TBM (Tris Buffered Medium) containing 1 mM caffeine and 0.1% BSA (Sigma Co., A7888), and then 15～20 oocytes were placed in 50-μl drops of the fertilization medium that had been covered with warm paraffin oil in 60 Φ petri dish (Falcon Co. 1007). Semem was washed one time by centrifugation at 800 × g for 5 min in TL-HEPES, and precipitated sperm were swum up

in Sp-TALP (Sperm Tyrode-lactate medium). The concentration of suspending spermatozoa was measured by hemacytometer. Highly motile spermatozoa were added to Sp-TALP at a final concentration of 1.0×10^5 spermatozoa/ml. This sperm suspension was added to 1~2 μl of the medium that containing oocytes. Oocytes were incubated with spermatozoa at 39℃ in an atmosphere of 5% CO_2 in air.

3. Preparation of Spermatozoa and Sperm Heads

Porcine spermatozoa were separated from porcine semen and washed once by centrifugation at 800×g for 5 min in TL-HEPES supplemented with 0.1% BSA (Sigma;, fraction V) and then resuspended in 1.5ml of TL-HEPES. To obtain sperm heads, sperm suspension was sonicated in 3 ml of TL-HEPES medium. The sonication was conducted in water bath for 1 min using 100% output from ultrasonic sonicator(Branson 8210R-DTH model, USA). Then, the sonicated sperm suspension was centrifuged for 5 min at 1000×g to isolate sperm heads, and isolated sperm heads were washed once more in 1 ml of TL-HEPES by centrifugation consistent.

4. Removal of Acrosomal Membrane and Isolation of Perinuclear Theca

To remove acrosomal membrane, spermatozoa and isolated sperm heads were suspended in 0.02% Triton X-100 and agitated for 40 min. For the freezing and thawing, suspension of spermatozoa or sperm heads were dispensed into PCR tube. Each vial was placed directly into LN_2 (−196℃) and then vials were placed in 24~26℃ water or air for about 10 min according to method described by Wakayama et al (1998). After agitation, sperm suspension was washed with TL-HEPES by centrifugation.

5. Transfection

pEGFP-N1 Vector (Clonetech, USA) was amplified in DH5 α competent cells. DNA was isolated using Miniprep kit (Promega, USA) according to the manufacturer's protocol and linearized by EcoO 109 I restriction enzyme. As shown in Fig. 1, the size of the DNA used was 4.7 kb, containing the genes encoding EGFP and neo under the regulation of separate promoters. Nine microliter of membrane -disrupted sperm or sperm head suspension was mixed with 1 μl of linearized pEGFP-N1 in concentration of 5 ng/μl. The DNA-sperm mixture was incubated at room temperature for 1 min.

6. Injection of Spermatozoon and Sperm Head into Oocytes

Spermatozoa and sperm heads were resuspended in mixture of TL-HEPES and 7% polyvinylpyrrolidone (PVP) solution at 1 : 1 ratio. About 5~9 μl of this suspension was dropped on a lid of microinjection plate (Falcon 1006) and covered with mineral oil. The plate was placed on inverted microscope (Olympus CK40, Japan) with a Narishige micromanipulator (onm-1; Narishige, Tokyo, Japan). Oocytes were freed from cumulus cells by repeated pipetting in 0.1% hyaluronidase. The injection of spermatid into the oocyte cytoplasm was performed according to the method of Lee et al (1998). Immediately after injection, the micropipette was withdawn quickly, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocytes. After the injection, the oocytes were transferred to NCSU23 medium and cultured at 39℃ in 5% CO_2 in air, and the expression of EGFP was monitored under fluorescent microscope (BX60F3; Olympus, Japan).

7. Statistical Analysis

The data from at least four replications were pooled. Differences in the percentages of oocytes

developed to particular stage were determined by student t-test.

Ⅲ. RESULTS

1. Development of Porcine Embryos Injected with Spermatozoon or Sperm Head

Porcine oocytes injected with membrane removed spermatozoon or sperm head were cultured *in vitro* to blastocysts. As shown in Table 1, the rates of embryos developed to blastocysts in sperm and sperm head injection group were 11.6 and 5.1%, respectively, with significant differences (P<0.05). According to this result, spermatozoon was injected into oocytes in the following experiments.

Next, the effect of various treatment on spermatozoa prior to microinject into oocytes on the development of embryos was investigated. For this purpose, intact, freeze-thawed and Triton X-100 treated spermatozoa were injected into oocytes. The rates of embryos developed to blastocysts in embryos injected with intact, freeze-thawed and Triton X-100 treated spermatozoa were, 15.0%, 0.0% and 14.2% respectively. The developmental rate of embryos injected with freeze-thawed spermatozoa was significantly lower than those of other groups (Table 2).

2. Expression of EGFP Gene in Porcine Embryos.

Porcine spermatozoon or sperm head was injected into oocytes after Triton X-100 treatment and transfection of EGFP gene *in vitro*, and then investigated the expression of EGFP gene in the embryos. As shown in Table 3, expression rate, 42.1% of sperm injected embryos was significantly higher than that, 20.2% of sperm head injected embryos (P<0.05).

In the next experiment, the effect of treatment on spermatozoa on the expression of EGFP gene was investigated. As shown in Table 4, the expression rate of EGFP gene in embryos injected with freeze -thawed and Triton X-100 treated spermatozoon were 38.4 and 22.4%, respectively. The former was significantly higher than the latter.

The effect of EGFP gene concentration on the expression of EGFP gene in embryos was also

Table 1. Development of porcine embryos following injection of sperm or sperm-head

Treatment on sprem	Part of sperm	No. of injected oocyte	No. of cleaved embryo (%)	No. of blastocyst (%)
Triton X-100	Sperm	284	214 (75.3)	25 (11.6)[a]
	Sperm head	300	195 (65.0)	10 (5.1)[b]

[a,b] Values with different superscripts within column differ significantly(P<0.05).

Table 2. *In vitro* development of porcine embryos injected with sperm after various treatment

Treatment		No. of oocyte	No. of cleaved embryo (%)	No. of blastocyst (%)
ICSI	Control*	126	80 (63.5)	12 (15.0)
	Freeze & thaw	108	38 (35.1)	0 (00.0)**
	Triton x-100	100	63 (63.0)	9 (14.2)

* Intact sperm

** Values with different superscripts within column differ significantly (P<0.05).

Table 3. EGFP gene expression of porcine embryo following injection of sperm or sperm-head transfected with EGFP gene

Treatment on sperm	Part of sperm	No. of injected oocytes	No. of cleaved embryos (%)	No. of embryos expressed EGFP (%)
Triton X-100	Sperm	168	114 (67.0)	48 (42.1)*
	Sperm head	134	80 (59.7)	16 (20.0)

* Values with different superscripts within column differ significantly (P<0.05).

Table 4. EGFP expression in porcine embryo injected with sperm after various treatment

Treatment on sperm	No. of oocytes	No. of cleaved embryos (%)	No. of embryos expressed EGFP (%)
Control*	168	90 (53.5)	0 (0)
Freeze & thaw	150	52 (34.7)	20 (38.4)[a]
Triton X-100	196	98 (50.0)	22 (22.4)[b]

* Intact spermatozoa cultured with EGFP gene

[a,b] Values with different superscripts within column differ significantly (P<0.05).

investigated. When the concentration of EGFP gene in culture medium were tested as 0.01, 0.1 and 1.0 ng/ml, the expression rates were ranged from 22.2 to 37.4 and 18.5%, respectively, with significant differences among the different concentrations (Table 5).

Table 5. Effect of EGFP gene concentration on EGFP expression in porcine embryos

Treatment on sperm	Concentration of EGFP DNA	No. of injected oocytes	No. of cleaved embryos (%)	No. of embryos expressed EGFP (%)
Triton X-100	0 ng	120	88(73.3)	0 (00.0)
	0.01 ng	120	90(75.0)	20 (22.2)[a]
	0.1 ng	140	96(65.7)	36 (37.4)[b]
	1 ng	180	108(60.0)	20 (18.5)[c]

[a,b,c] Values with different superscripts within column differ significantly (P<0.05).

Table 6. *In vitro* development of embryos expressing EGFP gene

Treatment on spermatozoa			No. of oocyte	No. of developmental competence of embryos		
				2~4 cell (%)	8 cell (%)	Blastocyst (%)
Triton X-100	Non-injected with EGFP DNA		50	41 (82.0)	17 (34.0)	7 (14.0)
	Injected with EGFP DNA	EGFP exp.	82	32 (39.0)	15 (46.8)	1 (3.1)*
		EGFP non-exp.		50 (69.7)	22 (44.0)	6 (12.0)

* Values with different superscripts within column differ significantly (P<0.05).

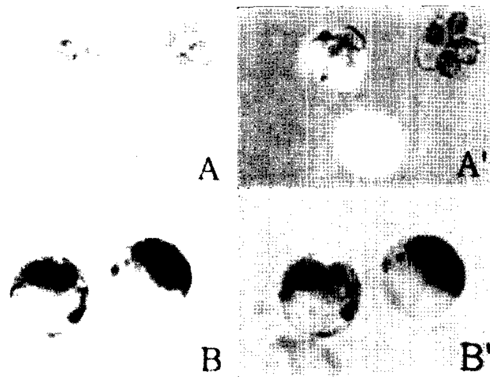

Fig. 1. Expression of EGFP-gene in porcine embryos. A and B; embryos observed under microscope (×200) and, A' and B'; embryos observed under fluorescent microscope (× 200). A, A' and B, B' are same embryos. A'; 4 cell embryos expressing mosaicism of EGFP, B'; Blastocysts mosaic expression of EGFP gene.

3. Developmental Competence of Porcine Embryos Transfected with EGFP Gene

As shown in Table 6, 3.1% of embryos expressing EGFP gene were developed to blastocyst. This result was significantly lower not only than that, 12.0% of non-expressing embryos but also that 14.0 % of control. These results suggested that integration and expression of EGFP gene in porcine embryos may bring harmful effect on developmental competence of the embryos.

The expression pattern of EGFP gene in embryos were investigated under fluorescence microscope. As shown in Fig. 1, EGFP gene integrated into embryos showed mosaicism.

Ⅳ. DISCUSSION

In the previous studies, it was reported that membrane-disrupted mouse spermatozoa supported the genomic integration and subsequent expression of exogenous DNA in developing embryos after ICSI (Perry et. al., 1999), and the transmission or introduced gene to the F_1 generation was reported by sperm mediated gene transfer (Lazzeresch et al., 2000). With the special reference to the above mentioned reports, in the present experiments, production of transgenic porcine embryos were attempted after cytoplasmic injection of spermatozoa transfected with EGFP gene. Plasma membrane of sperm or sperm head were removed to enhance the integration of foreign DNA into sperm or sperm head.

Following intracytoplasmic sperm or sperm head injection after removal of their acrosome membrane, 75.3% and 65% of embryos were normally fertilized. However, developmental rate to blastocyst stage was significantly higher in sperm injection group (11.6%) than sperm head injection group (5.1%) as presented in Table 1. According to this results, acrosome membrane removed spermatozoa were used in following experiments. When intact, frozen and thawed, and Triton X-100 treated spermatozoa were microinjected into oocytes, the developmental rates upto blastocysts were 15.0, 0.0, and 14.2%, respectively (Table 2).

These results indicate that frozen-thawed spermatozoa are inferior to Triton X-100 treated spermatozoa in supporting developmental competence of embryos. Similar trend obtained in the expression rate of EGFP gene in embryos microinjected with spermatozoa or sperm head. As shown in Table 3, expression rate of EGFP gene in embryos following microinjection of sperm or sperm head after transfection with EGFP gene were 42.1 and 20.0%, respectively, with significant difference. According to these results, membrane removed spermatozoa were used as DNA carrier in the next experiments.

Capability of spermatozoa to carry foreign DNA into oocytes was changed by the method of membrane disruption. Cleavage and developmental rate of embryo up to blastocysts were low in embryos injected with frozen-thawed spermatozoa than those

of embryos injected with Triton X-100 treated spermatozoa. However, the reverse was observed in the expression rate of EGFP gene (Tables 2, 4). These results reconfirm the previous report that high rate EGFP expression was obtained from the embryos injected with Triton X-100 treated spermatozoa (Perry et al., 1999). Low cleavage and EGFP expression rates of embryos microinjected with frozen-thawed spermatozoa were considered to be due to the physical damage of spermatozoa under the cyoprotectant free condition (Wakayama et al., 1998). Sciamanna et al., (2000) reported that low foreign DNA concentration affects the expression of the DNA in the embryos. Similar result was obtained in this experiment. The significantly higher expression rate of 37.4% was obtained from the embryo microinjected with spermatozoa cultured in 0.1 ng/μl of EGFP gene. The spermatozoa cultured in medium including 0.01 and 1.0ng/μl EGFP gene resulted in expression rates of 22.2 and 18.5%, respectively (Table 5).

Developmental rate of embryos expressing and non-expressing EGFP gene up to blastocysts were 3.1 and 12.0%, respectively (Table 6). This result suggested the possibility that EGFP gene integrated into oocytes may exert harmful effect on the development of embryo. However, the mechanism of harmful effect of EGFP gene on embryo development was not clear. Most of the embryos expressing EGFP gene showed mosaic type expression. This result suggested that foreign DNA remains as non-integrated form in the sperm nucleus (Kuznetsov et al., 2000). Therefore, it could be said that mosaic pattern of expression of exogenous DNA in the cytoplasm suggests that DNA was mostly bound on the sperm surface rather than inside the sperm.

In conclusion, in pig, membrane disrupted sperm could be integrated with exogenous EGFP gene and carry the gene into oocytes by microinjection of the spermatozoa. However, more basic studies must be carried out to produce transgenic pigs by this procedure.

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요 약

돼지에 있어서 난자내 정자 직접 주입에 의한 외래 유전자 도입에 관한 연구

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정자를 매개체로 한 유전자 전이는 형질 전환 동물의 생산을 위한 가능성 있는 간단한 방법이다. 또한 세포질내 정자 주입법에 의한 외래 유전자의 전이에 의한 형질 전환 동물의 생산이 최근에 보고되었다. 본 연구에서는 정자를 EGFP 유전자와 공배양한 후 이를 난모세포내에 미세 주입한 다음, 수정란의 발달과 EGFP 유전자의 발현을 조사하였다. 돼지 난자에 정자, 세포질막이 파괴된 정자 또는 정자를 주입하였다. 주입 후 수정된 난자는 NCSU23 배양액에서 배반포까지 배양하였으며, 배발생율과 EGFP 유전자의 발현을 연구하였다. 정자를 미세 주입한 결과 난할율은 67.0%로 정자두부를 미세주입한 난할율인 59.7%보다 높았고, 수정란의 EGFP 유전자 발현율은 각각 42.1와 20.0%로서 전자가 유의하게 높았다. 세포질막을 파괴하기 위해 다른 방법들로 정자를 처리하여 주입하였을 시 수정란의 배발생율에 영향을 주지 않았다. 정자, 세포막이 파괴된 정자를 주입하여 배반포 발생율을 조사한 결과, 15.0과 14.2%로서 유의차가 없었다. 동결융해하거나 Triton X-100으로 처리한 정자를 주입하여, EGFP 발현율을 조사한 결과, 동결융해 정자를 사용했을 때의 성적이 38.4%로 타군의 성적인 22.4%에 비해 유의하게 높았다. 미세 주입에 앞서 정자는 EGFP 유전자의 0.01 ng/μl 부터 1 ng/μl까지의 여러 농도로 배양되었다. 그러나 난할율을 조사한 결과 EGFP 유전자의 농도 차이에 따른 유의차가 없었다. 정자 배양액에 첨가된 EGFP 유전자의 농도가 0.1 ng/ml일 때 EGFP 발현율은 37.4%로서 가장 높은 결과를 보였다. 따라서 본 연구의 결과에 의하면 세포질막을 제거한 정자에 외래 유전자가 붙게 되며, 이 정자는 외래 유전자를 수정란내로 옮겨 매개체로서의 역할이 가능할 것으로 생각된다.

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