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Positive Expression of EGFP Gene in Bovine Embryos after ICSI using Spermatozoa Co-cultured with Exogenous DNA

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외래 유전자와 공배양한 정자를 이용해 난자내 직접주입술한 후 EGFP의 발현

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ABSTRACT

There are many methods to introduce exogenous DNA into embryo to produce transgenic animals. Exogenous gene can be integrated into oocyte by sperm vector. In this study, sperm was used as a vector for a transgene, which is encoding enhanced green fluorescent protein (EGFP). The objective of this study was to investigate the expression of exogenous gene in bovine embryos after injection of spermatozoa cocultured with EGFP DNA fragment. Spermatozoa were plunged into liquid nitrogen and thawed several times or shook in 0.2% Triton X-100 to remove sperm membrane followed by DTT treatment. The injected oocytes were co-cultured with vero cells in CR1aa, and expression of EGFP gene was observed under fluorescent microscope. Blastocyst formation rates of oocytes injected with sperm treated with DTT, DTT-freezing or DTT-Triton X-100 were 34.7, 39.4 and 31.9%, respectively. The rates of EGFP expression in oocytes injected with 54 ng DNA after DTT-treated, DTT-freezing and DTT-Triton X-100-treated sperm were 0, 19.1 and 13.9%. On the other hands, expression rate of oocytes injected with sperm cocultured with 13.5, 27 and 63.5 ng of EFGP DNA were 6.7, 9.0 and 5.1%, respectively. When intact sperm was mixed with 63.5 ng/μl EGFP DNA fragment, and then electroporated before injection, the expression rate of injected oocyte was 2%. Unexpectedly, electroporation could not increase the expression rate. These results suggest that sperm can be used as a transgene vector, even if the efficency was low (19.1%).

(Key words: Exogenous DNA, ICSI, EGFP)

Ⅰ. INTRODUCTION

There are several ways to produce transgenic animals; by microinjection (Gordon K., Ruddle F.H., 1986), using viral vectors or cloning (Campbell J., 1996). The general method is pronuclear microinjection of exogenous DNA into the male pronucleus of zygote. Unfortunately, transgene integration is usually a random and unpredictable event and the

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overall efficiency of this method is much lower in domestic species than in the mouse (Wall et al., 1996). There exist more effective techniques such as cloing with transfected cultured cells which is still difficult to achieve, or the use of retroviral vectors that often produce mosaic animals.

Viral-mediated gene transfer is possibly the original asexual gene transfer approach for mammals and one of the first gene transfer approach tried as a laboratory technique. Viral-mediated gene transfer has been successfully employed in many species; chickens (Brinster et al., 1991; Sargent, 1999), zebrafish (Lu et al., 1997) in addition to the original application in mice. More recently, retrovirus -mediated gene transfer has been used to produce transgenic cattle (Chan et al., 1998) and rhesus monkeys (Chan et al., 2001). The primary advantages of this approach are high frequency of gene transfer across embryonic membranes, high integration into oocyte/zygotic genome and minimal required embryo manipulation. However, retrovirus -mediated gene transfer method has some disadvantages. One of the most serious limitations of retrovirus gene transfer is the relatively small amount of genetic imformation (10 kb) that can be transported because of the physical limitation in volumn of the viral particles. The other potential disadvantage of retrovirus mediated gene transfer is the complexity of the process. Though "introducing" viral particles to oocytes requires the complicated embryo manipulation, packing transgenes into virions takes many steps.

Sperm mediated gene transfer (SMGT) seems to be an easier method to achieve transgenesis. Sperm cells are known to bind proteins and DNA in almost all species (Lavitrano et al., 1989). Recently, Perry et al. (1999) have shown that intracytoplasmic injection of 'pre-treated' sperm carrying exogenous DNA can be used to produce trangenic mice. Perry et al. (1999) developed a new gene transfer method (Pittoggi et al., 1999) and called his new apporach "MII transgenesis". This technique resulted in 19% transgenesis of 57 pups produced. Perry et al. (1999) found that it was neccessary to pretreat sperm with freezing and thawing or Triton X-100 to produce transgenic animals. Sperm-mediated gene transfer now has been demonstrated in a wide variety of species; cattle and chiken (Sin et al., 2000), golden hamster (Fernandez et al., 1999), Pig (Cappello et al., 2000; Qian et al., 2001; Seo et al., 2000), rabbit (Lavitrano et al., 1989), salmon (Solaiman et al., 2000), silkworm (Shemech et al., 2000), shellfish (Vassiler et al., 2001), and zebrafish (Kuznetsov et al., 2000).

With these reports, this study reconfrimed the effects and exogenous gene expression rates of sperm treatment. The objective of this study was to produce transgenic bovine embryos by ICSI using spermatozoon co-cultured with exogenous DNA.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* Maturation of Follicular Oocytes

Collected oocytes were washed three times with Tyroide-Hepes medium (TL Hepes; Parrish et al., 1985) and cultured in tissue culture medium (TCM -199; Gibco BRL Co., USA) supplemented with 2.2 g/ℓ sodium bicarbonate, 10% (v/v) heat-treated fetal bovine serum (FBS; Gibco), 0.22 μl/ml FSH (Follitropin V; Vetrepharm, Canada) and 1 μl/ml estradiol-17β(Sigma Co., St. Louis, MO). The oocytes were cultured in 50 μl drops of TCM-199 under mineral oil (Sigma) for 20 hr at 39℃, 5% CO_2 in humidified atmosphere.

2. *In Vitro* Fertilization (IVF)

In vitro fertilization was carried out as described in Sirard et al. (1988). Matured oocytes were washed twice with Sp-TALP and then with Fert-TALP (Rosenkrans et al., 1991). After washing, ten mature

oocytes were pulled into 44 μl Fert-TALP droplets under paraffin oil. Bull spermatozoa recovered from frozen-thawed semen were seperated in a discontinous percoll gradient. Highly motile spermatozoa were added to final concentration of 1.0×10^6 sperm /ml. Then 2 μl of heparin stock solution (2 mM phenicillamine, 20 μM hypotaurine and 1 μM epinephrine) to stimulate sperm motility were added into Fert-TALP droplets. The fertilization media were incubated at 39℃, 5% CO_2 in humidified atmosphere.

3. Sperm Preparation for Intracytoplasmic Sperm Injection (ICSI)

The spermatozoa that were used for this experiment were from the frozen semen of a bull. One of 0.25 ml straw of frozen semen was thawed in water at 37℃ for 1 min, and the contents of the straw were placed on a discontinous Percoll (Sigma) gradient. The gradient consisted of frozen-thawed semen layered over 2 ml of 45% Percoll and 2 ml of 90% Percoll in a 15 ml conical plastic tube. After centrifuge at 1500g for 15 min, the sperm pellet was then resuspended in fertiliaztion medium supplemented with 6 mg/ml bovine serum albumin (BSA, Sigma) and 1 mg/ml gentamycin. In a small culture tube, a 0.1 ml aliquot was layered under 0.8 ml fertilization medium supplemented with 0.1 ml of 5 mM DTT (Sigma). After 1 hr of incubation, the spermatozoa that had sum to the top 0.5 ml in the culture tube were washed twice by suspension and centrifugation at 350×g for 10 min in 10 ml fertilization medium to remove the DTT. The sperm pellet was resuspended with 1 ml fertilization medium and these sperm cells were used for the treatment.

4. Removal of Acrosomal Membrane

To remove acrosomal membrane, isolated sperm cells were shaked with 0.2% Triton X-100 (Sigma) for 40 min or frozen and thawed. After agitation, sperm suspension was washed with TL-HEPES medium by centrifugation.

5. Sperm Microinjection

Sperm injection was perfomed using Olympus CK40 inverted microscope with a Narishige micromanipulator (ONM-1, Narishige, Tokyo, Japan). Sperm pellet was diluted approximately 1 : 2 with 5% polyvinylpyrrolidione (PVP, MW 366,000; Sigma) in TL-HEPES stock.

The drops for manipulating work consist of two round PVP droplets (10 μl), sperm droplet (20 μl) and one elongated oocyte droplet (30 μl), and those were placed on the center of dish (Falcon #1006) and coverd with mineral oil. Denuded oocytes were placed in the elongated droplet of HEPES buffer. In the PVP drop, injetion pippet aspirated PVP mixture and then individual spermatozoon was picked up into injection pippett. The injection pippet was moved to oocyte droplet. An oocyte was held by holding pippet at the 9 o'clock position, in the meantime, the polar body was at either 6 or 12 o'clock position. After the injection pippet containing a spermatozoon was inserted into the ooplasm at 3 o'clock, a moderate vacuum was established in order to break the ooplasma and suck some of the ooplasm into the injection pippet. Subsequently, the aspirated ooplasm and spermatozoon were expelled into the ooplasm with minimum volumn of medium. The sperm suspending medium was retrieved to the extent possible, with great care being taken not to extract an excessive amount of ooplasm.

6. Oocyte Activation and Culture

Injected oocytes were activated by ionomycin (Sigma) and 6-dimethylaminopurine (DMAP). At First, Injected oocytes were washed three times in CR1aa (supplemented with 3 mg/ml fatty-acid free BSA, 20 μl/ml, MEM essential amino acid, 10 μl/ml MEM non-essential amino acid, 0.44 μl/ml Na

pyruvate, 1.46 μl/ml glutamine and 25 μl/ml gentamycin). Oocytes exposed to 5 μM ionomycin for 8 min at 39℃ were rinsed in CR1aa supplemently with BSA to stop activation, then exposed to 1.97 mM DMAP for 4 hr (Susko-Parrish et al., 1994). Activated oocytes were co-cultured with vero cells for 8 days. Embryo development was assessed with an inverted microscope at 12 hr intervals up to 192 hr after injection.

7. Preperation of DNA

pEGFP-N1 encodes a red-shifted variant of wild -type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maximum = 488 nm; emission maximum = 507 nm). DNA was isolated using Maxiprep kit (Promega, Madison, WI) according to the manufacturer's protocol and linearized by EcoO109 I rectriction enzyme. As shown in Fig. 1, the size of the DNA used were 4.7 kb containing the genes encoding EGFP and neo^R under the regulation of seperate promoter.

8. Co-incubation of Sperm with Linearized EGFP DNA

5 μl of membrane-disrupted sperm suspension containing 1×10^5 spermtozoa in HEPES buffer were mixed with 5, 10, 20 and 25 μl of linearized EGFP -N1 DNA to give a final DNA concentration of 13.5, 27, 54 and 63.5 ng/μl. The DNA-sperm mixture was incubated at 37℃ for 5 min.

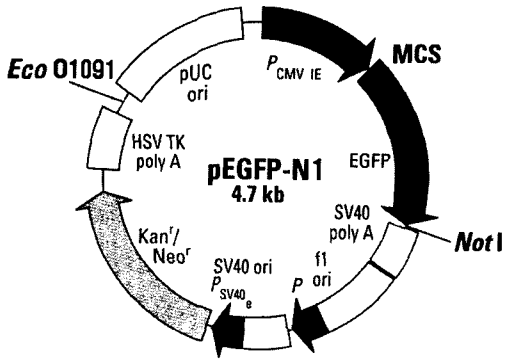

Fig. 1. pEGFP-N1 vector containing the gene encoding enhanced green fluorescent protein (EGFP).

9. Co-incubation of Sperm with Linearized EGFP DNA for Electroporation

5 μl of intact sperm suspension containing of 1×10^5 spermatozoa in HEPES buffer were mixed with 25 μl of linearized EGFP-N1 DNA to give a final DNA concentration of 63.5 ng/μl. The DNA-sperm mixture was incubated at 37℃ for 5 min before electroporation and then, electroporated with 300 V for 99 μsec.

10. Statistical Analysis

The data from at least four replications were pooled. Differences in the percentages of oocytes developed to the particular stage were determined by Chi-square analysis.

Ⅲ. RESULTS

Table 1 shows the comparision of IVF and ICSI, which can clarify the effect of ICSI. Bovine oocytes were injected with decondenced spermatozoon. *In vitro* developmental potential of embryos injected with sperm treated with DTT was compared with that of *in vitro* fertilization (IVF)

Table 1. *In vitro* development of bovine oocytes after ICSI and IVF

Fertilization method	No. of oocytes injected	Developmental stage	
		Oocytes cleaved(%)	Blastocyst (%)
IVF	109	88(80.7)	27(30.6)
ICSI	133	95(71.4)	33(34.7)

The difference between groups were not significantly (P>0.05).

oocytes (Table 1). The rates of oocytes developed to blastocysts in ICSI and IVF were 34.7 and 30.6%, respectively, but there was no significant differences.

In the next step, the effect of various treatment of spermatozoa prior to microinjection into oocytes on the development of oocytes was investigated. By DTT treatment, sperm head was decondensed. This procedure was conducted immitating the nomal fertilization which sperm head chromatin was fused with oocyte chromatin after decondensation. (Anthony., Perry et al., 1999). As shown in Table 2, the cleavage rate after ICSI with sperm treated with DTT was similar to (83.3%) that of after artificial activation group (control). Blastocyst formation rates of oocytes injected with sperm treated by DTT, freezing and thawing and Triton X-100 were 37.8, 39.4 and 35.0%, respectively. There were no significant differences in developmental rate after injection with treated spermatozoa.

In order to wether EGFP expression level is dose -dependent or not, we treated with different concentration of EGFP DNA fragmant such as 13.5, 27, 54 and 63.5 ng, after freezing and thawing with DTT treatment. The expression levels were 6.7, 9.0, 15.4 and 5.1%, respectively. As shown in Table 3, the expression rates were enhanced from 13.5 ng to 54 ng, however, the expression rate was decreased when treated with 63.5 ng of EGFP DNA (5.1%). Therefore 54 ng of EGFP concentration was used for further experiment.

Expression levels of EGFP DNA fragment were examined, following ICSI with transfected bull sperm. As expected, EGFP DNA was expressed in

Table 2. *In vitro* development of bovine oocytes injected with sperm treated by various methods

Sperm treatment	No. of oocytes injected	No. of oocytes cleaved (%)	No. of blastocysts (%)
Control*	176	152(86.3)	54(35.5)
DTT	142	119(83.8)	45(37.8)
DTT, Freezing & Thawing	183	157(85.7)	51(32.4)
DTT, Triton X-100 0.2%	171	137(80.1)	48(35.0)

The difference between groups were not significantly (P>0.05).
*control; artificial activation by ionomycin and 6-DMAP.

Table 3. Effect of EGFP gene concentration on EGFP gene expression in bovine embryos

Sperm treatment	Concentration of EGFP DNA (ng)	No. of oocytes injected	No. of embryos cleaved (%)	No. of EGFP embryos expressed (%)
Freezing & Thawing	Control*	89	67(75.3)	N/A**
	13.5	60	30(50.0)	2(6.7)[a]
	27	68	44(64.7)	4(9.0)[b]
	54	92	71(77.1)	11(15.4)[c]
	63.5	83	59(71.0)	3(5.1)[a]

[a,b,c] Values with different superscripts within colume differ significantly (P<0.05).
* Control; artificial activation by ionomycin and 6-DMAP.
** N/A; not applicable.

Table 4. Expression of EGFP following ICSI with transfected bull sperm

Sperm treatment	No. of oocytes injected	No. of oocytes cleaved (%)	No. of blastocysts (%)	No. of embryos EGFP expressed (%)
Control*	212	182(85.8)	68(37.3)	N/A**
DTT	179	151(84.3)	57(37.7)	0(0.0)[a]
DTT, Freezing & Thawing	224	188(83.9)	74(39.4)	36(19.1)[b]
DTT, Triton X-100 0.2%	252	208(82.5)	76(36.5)	29(13.9)[b]

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

* Control; artificial activation by ionomycin and 6-DMAP.

** N/A; not applicable.

all groups except for the DTT treatment only. Table 4 showed that the highest expression levels were detected in the oocytes injected with frozen-thawed sperm transfected with EGFP DNA.

The spermatozoa in this study, were pretreated by freezing and thawing after decondensation with DTT. These pictures show mosaic expression of EGFP DNA after ICSI with sperm simply incubating with 54 ng of EGFP DNA.

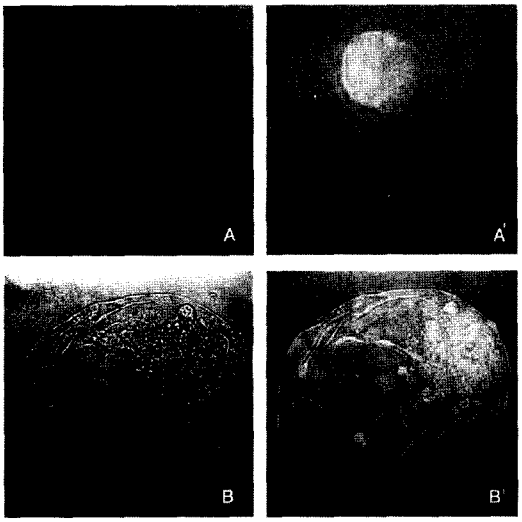

Fig 2. Expression of EGFP-gene in bovine embryos. A and B; 2-cell and hatched embryos observed under microscope (× 200). A' and B'; 2-cell and hatched embryos expressing EGFP-gene observed under fluorescent microscope (×200).'

In Table 5, we investigated how much exogenous DNA integrated into the embryo can influence on the embryonic development rate. The embryos were divided into two groups (expressed group of EGFP DNA and non-expressed group of EGFP DNA). Expressed group of EGFP DNA showed 0% of blastocyst formation, however, non-expressed group of EGFP DNA showed 29.5% of blasstocyst formation. Therefore exogenous DNA may have the negative effect against the embryonic development rate.

There are methods for enhancing expression level of EGFP DNA such as calcium phosphate, electroporation and liposome. Among them in this study, electroporation was used for enhancing method. Spermatozoa were electroporated with EGFP fragments to increase the expression level. Unexpectedly, ICSI with electroporated sperm (300 V, 1 time, 99 μsec) did not show the enhanced expression levels though it was not related to the development rate.

Ⅳ. DISCUSSION

In the present study, the effect of various sperm treatment on developmental potential and exogenous gene expression were investigated by using ICSI. ICSI has been utilized in different mammalian species such as hamster (Uehara & Yanagimachi., 1976), rabbit (Hosoi et al., 1988; Keefer et al.,

Table 5. Comparison of *in vitro* development between EGFP expression and non-expression groups

Treatment on spermatozoa		No. of oocytes	No. of developmental competence of embryos		
			2-4cell (%)	No. of morula (%)	No. of blastocysts (%)
Control*		68	53(77.9)	21(30.8)	17(32.0)[a]
Freezing & Thawing	EGFP expression	93	16(17.2)	3(18.8)	0(0.0)[b]
	EGFP non-expression		44(47.3)	15(34.0)	13(29.5)[a]

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

* Control; artificial activation by ionomycin and 6-DMAP.

Table 6. Expression of EGFP followed by ICSI with electroporated bull sperm

Sperm treatment	No. of oocytes injected	Developmental stage		
		No. of oocytes cleaved (%)	No. of blastocyst (%)	No. of embryos expressed (%)
Control*	105	81(77.1)	28(34.5)	N/A**
Electroporated sperm***	124	100(80.6)	29(29.0)	2(2.0)

* Control; artificial activation by ionomycin and 6-DMAP.

** N/A; not applicable.

*** electroporation; 300 V, duration 1 time, 99 μsec.

1989), cattle (Goto et al., 1992; Mansour et al., 1995), mouse (Ahmadi et al., 1995; Kuretake et al., 1996), horse (Squires et al., 1996) and sheep (Catt et al., 1996). In cattle, immotile sperm or isolated sperm heads have been used for ICSI which the successful viable blastocysts and live young were produced (Goto et al., 1990; Goto., 1993; Ki et al., 1993; Hamano et al., 1999). However, the developmental potential to blastocyst was very low in livestock (Keefer et al., 1989; Goto et al., 1990; Goto., 1993; Catt et al., 1996). In this study, decondensed sperm by DTT was used to induce similar phenomenon that of *in vitro* fertilization (Perry et al., Anthony C.F., 1999), and this procedure is essential for ICSI, in every group of sperm treatment except for electroporation group.

The experiment for examination of effect in varios sperm treatment, blastocyst formation rate was 30.6% in IVF and 34.7% in ICSI with DTT treated sperm. These results showed that there were no significant difference IVF and ICSI. Therefore ICSI can be a useful reproductive technique in cattle. In this study, artificial activation groups were as control groups in Table 2, Table 3, Table 4, Table 5 and Table 6. Activation can be induced by a variety of stimulation including exposure to calcium ionophore (Ware et al., 1989), ethanol (Nagai et al., 1987), electric shock (Ware et al., 1989), cycloheximide and 6-DMAP (Fulka et al., 1991). In this study, activation was performed by 5 μM ionomycin and 1.97 mM 6-DMAP. Most oocytes (80%) activated by ionomycin/6-DMAP were cleaved after 28 hr postactivation. This suggested that activation procedure applied in this experiment worked efficiently. To make easy to attach the EGFP DNA on the sperm head, removal of sperm

membrane is needed. Disrupting the sperm membrane by freezing and thawing before injection (Goto et al., 1990; Wakayama et al., 1998) or shaking with Triton X-100 (Ollero et al., 1998) have been reported to raise attachment of foreign DNA (Anthony C.F. et al., 1999). In this study, both of them were used, thus freezing and thawing and Triton X-100 showed similar expression levels (Table 4), freezing and thawing was picked up as a simple method for removal of sperm membrane. However, cleavage and developmental rate of embryo up to blastocyst were not different between freezing and thawing and Triton X-100 (Table 2). To Optimize concentration of EGFP DNA, decondensed spermatozoa were co-cultured with 13.5, 27, 54 and 63.5 ng/μl before injection. The Highest EGFP DNA expression level was obtained when the oocyte was injected with sperm co-cultured with concentration of 54 ng/μl EGFP gene. Up to 54 ng/μl of EGFP gene, the expression levels increased, however, over 54 ng/μl of EGFP gene, expression levels tended to decrease, significantly (Table 3). Thereforem in this study 54 ng/μl of EGFP DNA concentration was decided to be a optimal concentration. This result was in accordance with the previous reports that the settlement of appropriate DNA concentration 'not too high and not too low' is important (Shemesh M., Gurevich M., 2000).

According to the recent report, exogenous DNA may influent negative effect on the embryonic development level. To reconfirm this, the embryos which were produced contemporary experiment were divided into two groups, EGFP expressed group and EGFP non-expressed group. As showed in Table 5, EGFP DNA expressed group showed 0% of blastocyst formation while the EGFP non-expressed group showed 29.5%, respectively. This suggests that exogenous DNA may influent harmful effect on the embryonic developmental stages (Maione B., Lavitrano M., 1998), however, the mechanism of harmful effect of EGFP gene on embryo development is not clear. There are many methods to enhance the expressed level of EGFP gene such as calcium phosphate, electroporation and liposome. Among them, electroporation was used in this study, however, EGFP DNA expression level did not reach the highest level that of DTT-freezing and thawing group (Table 4, Table 6).

In this experiment, most of the embryos expressing EGFP gene showed mosaic expression. This result suggested that foreign DNA remains as nonintegrated form in the sperm nucleus (Kuanetsov et al., 2000). Therefore, it may suggest that exogenous DNA was mostly bound to sperm surface rather than integrated to sperm nucleus. As mentioned before, these results suggested that membrane disrupted sperm could carry the foreign gene into oocytes by microinjection of the spermatozoa though it showed mosaicism (Fig. 2). ICSI is simple method with low cost. The studies have shown that this can be accomplished *in vitro*, and further studies in live offspring will be needed to confirm efficient transgenesis by using this procedure.

V. 요약문

현재까지 외래 유전자를 도입하여 형질전환 동물을 생산하는 방법이 다방면으로 연구되어 왔다. 그 중에서 본 연구에서는 정자를 EGFP 유전자와 공배양한 후 이를 난모 세포내에 미세 주입한 다음, 수정란의 발달과 EGFP 유전자의 발현을 조사하였다. 즉, 동결후 융해나 Triton X-100 처리 등으로 세포막을 파괴하여, 이들 정자를 EGFP 유전자와 다분간 공배양함으로써 정자와 EGFP 유전자와의 결합을 유도하였다. 정자나 정자두부의 미세주입에 의해 수정된 난자는 0.3%의 BSA가 첨가된 CR1aa 배양액에서 배양하였으며, EGFP 유전자의 발현은 형광현미경 하에서 관찰하였다. 동결 후 융

해로 처리된 정자와 Triton X-100 처리 한 정자를 미세주입한 결과 난할율은 85.7과 80.1%였고, 배반포 발생율은 32.4 과 35.0%로서 유의차가 없었다. 동결 후 융해와 Triton X-100 으로 처리된 정자를 각각 미세주입한 수정란의 EGFP 유전자 발현율은 각각 19.1과 13.9%로서 전자가 유의하게 높았다. 또 정자 배양액에 첨가된 EGFP 유전자의 농도가 54 ng/μl일 때 EGFP 발현율은 15.4% 로서, 27 ng/μl일 때의 9.0%와 63.5 ng/μl일 때의 5.1%보다 유의하게 높았다. 발현율을 높히기 위한 방법 중 하나로써 electric shock의 방법을 이용해 보았으나 기존의 공배양 방법으로 얻은 최고 발현율인 19.1%에 못 미치는 2%를 보였다. EGFP 유전자가 발현된 수정란의 배반포 발생율은 0%로서 비발현 수정란의 29.5%보다 유의하게 낮았으며, EGFP 유전자의 발현은 mosaicism 형태를 보였다.

본 연구에서는 비록 낮은 외래 유전자 도입율을 보이기는 하나 (19.0%), 정자를 매개로 한 형질전환 동물의 생산은 그 방법이 간단하고 비용이 적게 든다는 장점이 있다. 기존 보고들의 효율성을 재고하여 볼 때, 난자내 정자 직접 주입술에 의한 형질전환 동물 생산의 연구는 향후 밝은 전망을 시사하고 있다.

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