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Expression of EGFP in Bovine Embryos after Intracytoplasmic Sperm Injection using Spermatozoa Co-cultured with Exogenous DNA

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ABSTRACT

This study was to investigate the expression of transgene after co-injection of spermatozoon and EGFP gene into mature oocytes in cattle. From frozen semen, spermatozoa were treated by DTT with 0.03% Tween-20, freezing and thawing or 0.02% Triton X-100 to disrupt their plasma membranes. The sperm injected oocytes were co-cultured with bovine oviduct epithelial cells in CR1aa, and expression of EGFP in embryos were observed under epifluorescent microscope. Two pronuclei were formed in oocytes injected with sperm treated by DTT (44.6%), DTT-Tween-20 (48.4%), DTT-freezing (44.4%) and DTT-Triton X-100 (42.9%). Cleavage and blastocyst formation rates of bovine oocytes which injected with sperm treated by DTT, DTT-Tween-20, DTT-freezing, and DTT-Triton X-100 were 49.1, 58.5, 43.9, and 48.4% and 10.2, 13.0, 6.8, and 6.5%, respectively. Although the most of embryos were showing mosaicism, embryos expressing EGFP gene were observed in all treated groups. Therefore, these results indicate that membrane disrupted sperm could interact with exogenous DNA, and that this procedure may be useful to introduce foreign gene into bovine oocytes.

(Key Words : Exogenous DNA, Spermatozoa, ICSI, EGFP, Bovine)

Ⅰ. INTRODUCTION

Sperm nuclear decondensation and male pronuclear formation are the first events after sperm-oocyte fusion that permit male contribution to the embryonic genome (Laurincik et al. 1998). There are many approaches that have been tried to study intracytoplasmic sperm injection (ICSI) during early mammalian embryo genesis such as hamster (Uehara et al., 1976), mouse (Kimura et al., 1995; Kuretake et al., 1996), rabbit (Hosoi et al., 1988), cattle (Goto et al., 1990) and human (Palermo et al., 1992). ICSI can be a valuable research tool for studying fundamental aspects of how the two gametes interact during fertilization.

Bull sperm nuclei were rarely decondensed in the hamster oocyte unless they have been treated with

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dithiothreitol (DTT) before injection (Perreault et al., 1988). Other disulfide-reducing agents (Calvin et al., 1971), anionic detergent, proteinase (Perreault et al., 1982) and high or low concentrations of salts (Rodriguez et al., 1985) also promote the decondensation of nuclear chromatin in mammalian spermatozoa. Therefore, sperm nuclear stability is considered to account for sperm decondensation and pronucleus (PN) formation occur following ICSI.

Several methods for the modifying mammalian genomes to integrate foreign DNA sequence transmissible through the germ line are reported. A method for microinjection of recombinant DNA into a pronucleus of a zygote was developed in the mouse in the early 1980s (Gordon et al., 1980). This method does not yet permit the transgene insertion to be controlled or predicted because of the quasi-random nature of integration site and number of copies integrated into the host genome (Gordon et al., 1980).

Problems in the available methods to produce transgenic animals have pushed a search for alternative methods, including the use of recombinant retrovirus to infect oocytes or preimplantation embryos (Chan et al., 1998) or replication-deficient adenovirus-mediated delivery systems (Kanegae et al., 1995) and spermatozoa as vehicles for DNA delivery during IVF (Lavitrano et al., 1989). Mouse sperm cells incubated with naked DNA could serve as vectors for introducing exogenous DNA into ova and producing transgenic mice. But the efficiency of transgenesis achieved in these studies has been still controversial (Lavitrano et al., 1989; Maione et al., 1998; Huguet and Esponda, 1998).

Recently, Perry and colleagues (1999) reported that ICSI of "damaged" sperm encoding exogenous DNA can be used to produce transgenic mice with a high efficiency. Since such procedure does not require zygote with visible pronuclei, it can be utilized as a good alternative method for the production of transgenic animals.

The objective of this study is to develop a method for the production of transgenic animal by using ICSI with membrane distrapted sperm that has perincubated with spermatozoa and foreign DNA and to improve the efficiency of producing transgenic animals. The expression rate of transgene EGFP after ICSI into bovine oocytes were investigated during the early embryo development.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation of Follicular Oocytes

Ovaries were obtained from the slaughterhouse and brought to the laboratory in saline at 37℃. The collected oocytes were washed three times in TL-HEPES medium (Parrish et al., 1985) and washed again three times in equilibrated TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 2.2 g/l sodium bicarbonate, 10% heat-treated fetal bovine serum (FBS; Gibco BRL), 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamycin sulfate, 1 μg/ml FSH (Folltropin V; Vetrepharm, Canada), and 1 μg/ml estradiol-17 β (Sigma Chmical Co., St. Louis, MO). Then oocytes were cultured in 50 μl drop of TCM-199 under paraffin oil for 24 h at 39℃, 5% CO_2 in humidified atmosphere.

2. *In Vitro* Fertilization

Matured cumulus-oocyte complexes (COCs) were washed twice with Sp-TALP and subsequently with Fert-TALP (Rosenkrans et al., 1993). After washing, ten mature COCs were pooled in a 44 μl of Fert-TALP droplet under paraffin oil. The spermatozoa that were used for this experiment were from the frozen semen of a bull. One 0.25ml straw of frozen semen was thawed in water at 37℃ for 10 sec, and the contents of the straw were placed on a discontinuous Percoll (Sigma) gradient.

Highly motile spermatozoa were added to Fert-TALP at a final concentration of 1.0×10^6 sperms/ml. Sperm concentration was determined with a hemacytometer. Then 2 μl of heparin stock solution to induce sperm capacitation and 2 μl of PHE stock solution (2 mM phenicillamine, 20 μM hypotaurine and 1 μM epinephrine) to stimulate sperm motility were added into a Fert-TALP droplet. Sperm and COCs were co-incubated in a 50 μl drops of Fert-TALP under paraffin oil for 24 h at 39℃, 5% CO_2 in humidified atmosphere.

3. Sperm Preparation for Intracytoplasmic Sperm Injection

The spermatozoa that were used for this experiment were from the frozen semen of a bull. One 0.25ml straw of frozen semen was thawed in water at 37℃ for 1 min, and the contents of the straw were placed on a discontinuous Percoll (Sigma) gradient. The gradient consisted of frozen-thawed semen layered over 2ml of 45% Percoll and 2ml of 90% Percoll in a 15ml conical plastic tube. After centrifuge at 857×g for 20 min, the sperm pellet was then resuspended in fertilization medium supplemented with 6mg/ml bovine serum albumin (BSA, Sigma) and 1 μg/ml gentamycin. In a small culture tube, a 0.1ml aliquot was layered under 0.8ml fertilization medium supplemented with 0.1ml of 5 mM DTT (Sigma). After 1 h of incubation, the spermatozoa that had sum to the top 0.5ml in the culture tube were washed twice by suspension and centrifugation at 350×g for 10 min in 10ml fertilization medium to remove the DTT. The sperm pellet was resuspended in 1ml fertilization medium and these sperm cells were used for sperm treatment.

To remove acrosomal membrane, isolated sperm were shook up with 0.03% Tween-20 (Sigma) for 30 min at 20℃ and with 0.02% Triton X-100 (Sigma) for 1 h. Freezing and thawing was conducted following previous method described by Wakayama et al. (1998). After agitation, sperm suspension was washed with fertilization medium by centrifugation.

4. Preparation of DNA

pEGFP-N1 Vector (Clontech, Palo Alto, CA) was transformed in DH5α competent cells. 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 = 488nm; emission maximum = 507nm). DNA was isolated using Maxi-prep kit (Promega, Madison, WI) according to the Promega's protocol and linearized by EcoO 109 I restriction. The size of the DNA used in this study were 4.7 kb, containing the genes encoding EGFP and neo^R under the regulation of each promoters.

5. Co-incubation of Sperm and Linearized EGFP DNA

Nine microliter of membrane-disrupted sperm suspension was mixed with 1 μl of linearlized pEGFP-N1 containing 5×10^5 sperm in fertilization medium to give a final DNA concentration of 7ng/μl the DNA-sperm mixture was incubated at room temperature for 1 min.

6. Oocyte Activation and Culture

Injected oocytes were washed twice in CRIaa (supplemented with 3mg/ml fatty acid-free BSA, 20 μl/ml, MEM essential amino acid, 10 μg/ml MEM non-essential amino acid, 0.44 μg/ml Na pyruvate, 1.46 μg/ml glutamine, 25 μg/ml gentamycin) and exposed 5 μM ionomycin (Sigma) in CRIaa medium for 5 min at 39℃. Activated oocytes were washed in CRIaa medium containing 1mg/ml BSA, cultured in CRIaa medium for 3 h to permit extrusion of their second polar body, then transferred to a drop of 1.9 mM 6-DMAP in CRIaa for

3 h. Activated ICSI oocytes were co-culture with bovine oviduct epithelial cells in 4-well dish (Nunc). These co-cultures were maintained for 7 days. Embryonic development was assessed with an inverted microscope at 12 h intervals for up to 7 days after injection.

7. Sperm Microinjection

Sperm injection was performed using a Olympus CK40 inverted microscope with a Narishige micromanipulator (ONM-1; Narishige, Tokyo, Japan). Sperm suspensions were diluted approximatly 1:2 with 5% polyvinyl pyrrolidone (PVP, MW 360 kd; Sigma) in 0.9% physiological saline. A low consisting of 2 round droplets and 1 elongated droplet was placed along the centerline of the dish (Falcon #1006). The first droplet (10 μl) was for washing (5% PVP), the second droplet (10 μl) was for the sperm suspension in PVP-saline, and the third elongated droplet (150 μl) was HEPES buffer medium for the oocytes. these droplets were covered with mineral oil. Denuded oocytes were placed in the elongated droplet. An individual sperm cell was picked up from the droplet of spermatozoa and PVP mixture by aspirating the tail first at the bottom of the dish, and then it was moved to the droplet containing the oocytes to be injected.

An oocyte was held to the holding pipette at the 9 o'clock position, with the polar body being at either the 12 or 6 o'clock position. After the injection pipette containing a spermatozoon was inserted into the ooplasm at 3 o'clock, a moderate vacuum was established in order to rupture the oolemma and suck some of the ooplasm into the injection pipette. Subsequently, the aspirated ooplasm and spermatozoon were expelled into the ooplasm with a minimum volume 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. One hour after injection, oocytes showing the spermatozoon in the perivitelline space were removed from the experiment.

8. Statistical Analysis

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

Ⅲ. RESULTS

In vitro development rates of bovine embryos after ICSI and IVF are summarized in Table 1. There were significant differences in developmental potential between ICSI and IVF embryos determined by cleavage to 2- or 4-cell stages at 48 h, and blastocyst stage at day 7 after fertilization. After ICSI, the developmental rate was lower than

Table 1. *In vitro* development after intracytoplasmic sperm injection(ICSI) and *in vitro* fertilization (IVF) in bovine oocytes

Fertilization methods	No. of oocytes injected	Developmental stage		Cell number of blastocysts (Mean±SEM)
		Cleaved oocytes(%)	Blastocyst(%)	
ICSI	108	53(49.1)[a]	11(10.2)[a]	114±1.83
IVF	110	91(82.7)[b]	32(29.1)[b]	118±2.30

[a,b] Within a column, values with different superscrips are significantly different ($p<0.05$).

those of IVF when the same sperm were used. When the number of nuclei of blastocysts derived from ICSI was compared with those of IVF-derived blastocyst in the fluorescent microscope after hoechst staining (Fig. 1), the average numbers of nuclei at the blastocyst stage following ICSI and

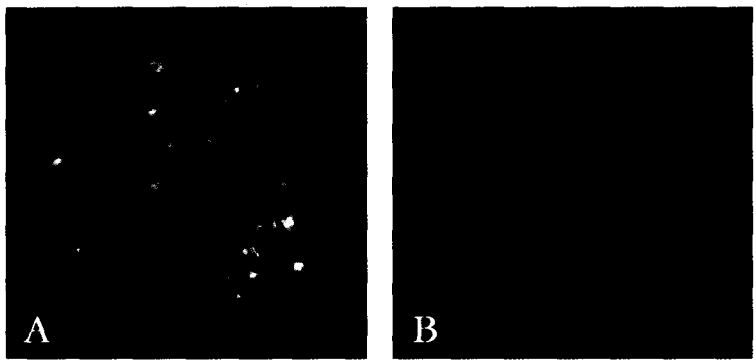

Fig. 1. Nuclear staining of bovine blastocyst following IVF and ICSI. Embryos were stained with Hoechst 33342; A) the blastocyst at day 7 after *in vitro* fertilization (IVF), B) the blastocyst at day 7 after intracytoplasmic sperm injection (ICSI). Original magnification : × 200.

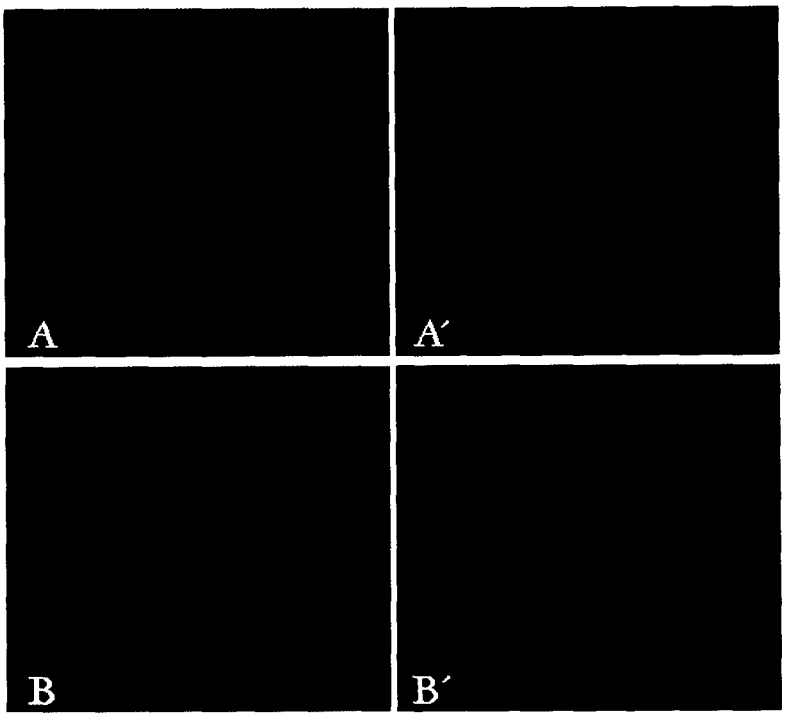

Fig. 2. Expression of EGFP in bovine embryos. A and B embryos observed under phase-contrast microscope × 200 and, A' and B' embryos observed under epifluorescent microscope × 200. A, A' and B, B' are same embryos. A') Mosaic expression of EGFP at around 8-cell stage embryo. B') Mosaic expression of EGFP at morula stage embryo.

IVF were 114 and 118, respectively, showing no significant difference between them.

The zygotes with two pronuclei and two polar bodies (2PN+2PB) were classified as normal fertilization at 18 h following ICSI (Table 2). The rates of formation of 2PN and 2PB were higher in DTT treatment than in normal sperm injection group (44.6 vs 5.8%). However, there were not significantly different between only DTT and other treatment groups (48.4, 44.4, 42.9%).

As shown in Table 3, the cleavage rate (49.1%) after ICSI using DTT treated sperm was higher than that (13.3%) of untreated sperm injected group (control). DTT treated sperm injected group was developed to the blastocyst stage (10.2%). However, oocytes injected with normal sperm were not developed to the blastocyst stage. No significant difference was noted in the cleavage and blastocyst formation rates between only DTT and other treatment groups.

Expression of EGFP was observed in all treated groups [DTT (3.8%), DTT-Tween-20 (11.1%), DTT-freezing (13.8%) and DTT-Triton X-100 (8.9%)]. However, none of the embryos in the untreated sperm injected group was expressed the EGFP. As a result of observation with fluorescent microscope, all of the EGFP expressed embryos showed mosaicism at 8-cell and morular stages (Fig. 2).

Ⅳ. DISCUSSION

In the present study, we demonstrated that EGFP expression of bovine embryos by ICSI of spermatozoa co-cultured with EGFP gene. Many investigators have tried to improve the ICSI technique in bovine. However poor *in vitro* development of the microinjected bovine oocytes has commonly been encountered. This problem may be related to the inconsistent level of sperm decondensation and subsequent pronuclear formation observed following ICSI in bovine.

Sperm nuclear structure was progressively condensed and stabilized by the formation of disulfide bonds (Calvin et al., 1973). Bovine sperm nuclei have particularly strong disulfide bond (Perreault et al., 1988), which could be expected to result in hyperstabilized chromatin, preventing the sperm nucleus from decondensation (Huret et al., 1986). In this study, incubation of bovine sperm with DTT was shown to lead to decondensation of the sperm nucleus and these treated sperm heads were showed the tight folding at near their equatorial segments (Rho et al., 1998). Treating the sperm with DTT before using them for ICSI clearly increased the rate at which they gave rise to male pronuclei in injected oocytes. The cleavage rate of DTT treated sperm injected group was approximately four times higher than that of untreated sperm injection group. None of the oocytes injected with untreated sperm continued development past the 16-cell stage (Table 3). These data might indicate that DTT can promote the decondensation of sperm

Table 2. Cytological observations at 18 h after intracytoplasmic injection with sperm treated with various agents

Sperm treatment	No. of oocytes injected	Formation of 2PN, 2PB (%)
Control	121	7(5.8)
DTT	130	58(44.6)
DTT, Tween-20	128	62(48.4)
DTT, Freezing	54	24(44.4)
DTT, Triton X-100	112	48(42.9)

Table 3. *In vitro* development of bovine oocytes injected with sperm of various treatments

Sperm treatment	No. of oocytes injected	No. of oocytes cleaved (%)	No. of oocytes blastocysts (%)
Control	90	12(13.3)	0(0.0)
DTT	108	53(49.1)	11(10.2)
DTT, Tween-20	123	72(58.5)	16(13.0)
DTT, Freezing	132	58(43.9)	9(6.8)
DTT, Triton X-100	93	45(48.4)	6(6.5)

Table 4. Expression of EGFP followed by ICSI with transfected bovine sperm

Sperm treatment	No. of oocytes injected	No. of oocytes cleaved (%)	No. of EGFP Exp. (%)
Control	90	12(13.3)	0(0.0)
DTT	108	53(49.1)	2(3.8)
DTT, Tween-20	123	72(58.5)	8(11.1)
DTT, Freezing	132	58(43.9)	8(13.8)
DTT, Triton X-100	93	45(48.4)	4(8.9)

head. Therefore, DTT treatment of the sperm were considered to the necessary process for bovine ICSI, but various treatments on sperm did not affect on the development rate of embryo after ICSI.

In bovine, treatment of spermatozoa with DTT to destabilize disulfide bonds prior to injection, combined with artificial oocyte activation after injection, resulted in improved rates of pronuclear formation and blastocyst development (Rho et al., 1998). While DTT treatment facilitated sperm processing in the activated oocyte, injection of DTT treated spermatozoa alone was not sufficient to stimulate oocyte activation. Activation can be induced by a variety of stimuli, including exposure to calcium ionophores (Ware et al., 1989), ethanol (Nagai, 1987), electric currents (Ware et al., 1989), cycloheximide and 6-dimethyl amino purine (Fulka et al., 1991). In this study, activation was performed with some modification(Susko et al., 1994). Injected oocytes were initially activated with 5 μM ionomycin, followed by 3 h culture period to allow extrusion of the second polar body, and incubated in 1.9 mM 6-DMAP for 3 h to complete the activation process.

Increased permeability of the sperm membrane by physical means may have a role to play in facilitating decondensation and PN formation after ICSI. Artificial removal of the acrosome by sonication (Keefer, 1989; Goto, 1993), damaging the sperm membrane by freezing and thawing before injection (Goto et al., 1990; Wakayama et al., 1998), crushing the sperm with the micropipette used for injection (Lacham-Kaplan et al., 1994), and shaking up with Tween-20 or Triton X-100 (Ollero et al., 1998) have been reported to improve sperm decondensation and PN formation. A longer time interval between sperm death and injection into the cytoplasm may result in disappearance of sperm cytosolic factor, and therefore poor fertilization. Sperm cytosolic factor is responsible for oocyte activation and embryonic development up to the blastocyst stage in the mouse (Swann and

Lawrence, 1996).

The role of the spermatozoon during fertilization includes the transfer of a haploid genome to the resultant zygote. This capacity has been exploited as an innovative method for the delivery of exogenous DNA for the production of transgenic animals (Perry et al., 1999). This study (1999) demonstrated that intracytoplasmic injection of membrane-distrupted-sperm carrying exogenous DNA could be used to produce transgenic mice with a high efficiency. They showed that membrane-disrupted mouse spermatozoa resulted in the genomic integration and subsequent expression of exogenous DNA in developing embryos after ICSI. An ICSI-based method has been previously described for the generation of Xenopus embryos in which sperm nuclei were first partially decondensed before mixing with DNA (Kroll and Amaya, 1996). Thus, these reports suggest notion that sperm submembrane components can be associated with exogenous DNA and promote transgenesis.

In this study, we showed that efficiency of transgene introduction in bovine sperm can be improved by the disruption of sperm membrane and subsequent ICSI. In addition, it was tested whether the decondensation of protamine by reducing disulfide bond might be ease the introduction of exogenous DNA into the genome of the sperm. However, the treatment of sperm with DTT before co-incubation with linearlized pEGFP-N1 was not affected by the expression of transgene. Therefore membrane disrupt process (Tween-20, freezing or Triton X-100) are an useful method to elevate EGFP gene transfer (Table 4).

The present data suggest that the pretreatment of sperm with DTT and the activation of oocytes with ionomycin and 6-DMAP are useful for facilitating sperm decondensation as well as subsequent PN formation after ICSI. In other word, membrane disrupted sperm can introduce the exogeneous DNA into oocyte by co-incubation with DNA, and that procedure may be a useful tool of produce to transgenic animal.

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요 약

소 난자에 있어서 외래유전자가 도입된 정자의 직접 주입에 의한 EGFP의 발현

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본 연구에서는 정자와 외래유전자인 EGFP유전자를 공배양한 후 정자직접 주입술로 난자를 수정시켜 EGFP유전자의 발현을 조사하였다. 정자는 외래유전자의 도입이 용이하도록 동결융해, 0.03% Tween-20과 0.02%의 Triton X-100의 처리를 통하여 정자두부의 원형질막을 제거하여 공시하였다. 수정된 난자는 소 난관상피세포가 포함된 CR I aa 배양액에서 공배양을 통하여 체외발달시켰으며, 난자의 발달에 따라 EGFP유전자의 발현을 형광 현미경 하에서 조사하였다. 원형질막이 제거된 정자로부터 수정란의 정상수정을 확인하기 위하여 18시간째 2PN 2PB를 조사한 결과, 발생율은 각각 DTT 처리구 44.6%, DTT와 Tween-20 처리구 48.4%, DTT와 동결융해 처리구 44.4%, 그리고 DTT와 Triton X-100 처리구 42.9%였다. 수정란의 초기 배 분할율은 DTT 처리구 49.1%, DTT와 Tween-20 처리구 58.5%, DTT와 동결융해 처리구 43.9% 그리고 DTT와 Triton X-100 처리구 48.4%였으며, 배반포 형성율은 DTT 처리구 10.2%, DTT와 Tween-20 처리구 13.0%, DTT와 동결융해 처리구 6.8% 그리고 DTT와 Triton X-100 처리구 6.5%였다. 이들 발달된 수정란 중 도입된 EGFP 유전자의 발현율은 DTT 처리구 3.8%, DTT와 Tween-20 처리구 11.1%, DTT와 동결융해 처리구 13.8% 그리고 DTT와 Triton X-100 처리구 8.9%로 나타났으며, 대부분의 발현은 모자이크 형태로 관찰되었다. 따라서 본 연구의 결과에 의하면 소에서 원형질막을 제거한 정자와 외래유전자의 공배양과 이 정자의 난자내 직접도입법에 의해 외래유전자를 가진 형질전환 소 수정란과 형질전환 소 생산이 가능할 것으로 생각된다.

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