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정자에 의한 외래 DNA의 계란내 도입: 유전자 변환 닭 생산을 위한 장애 극복

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Cracking Hen's Eggs for Transgenesis, without Cracking Them

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ABSTRACT

Hen's eggs have been regarded as one of the best animal bioreactors to produce biologically active peptides originated from many organisms including human. Despite the last decade's efforts to produce transgenic chicken for any commercial purposes, the results so far reported are very disappointing, indicating that hen's eggs are very difficult to crack for transgenesis. Comparatively large female gamete with enormous amount of yolk may be one of the major obstacles in achieving a similar feat to those of other vertebrate species including mouse, sheep, fish and frog. The delay or less efficiency evidenced may instruct to try an alternative way of gene transfer into chicken egg. Sperm-mediated gene transfer is one of them, and may require a great deal of understanding of mechanisms involved in early fertilization and embryonic development. In other animals where the technique was successful, basic mechanisms have been well studied and established only by painstaking efforts for decades. This paper discusses the accumulated knowledge on early fertilization mechanism in the chicken and how can this information be utilized to find the alternative gene transfer in making transgenic chicken.

(Key words : early fertilization, transfected sperm, chicken transgenesis)

INTRODUCTION

There have been various efforts to produce transgenic chicken using different techniques from those used in mammalian species(Bosselman et al., 1989). The techniques used include gene transfer using viral infection, direct DNA injection into the ova or embryo either with or without other mediating liposomal complexes, and injection of transfected cells into the host embryos. All these require exposing developing eggs to outer environment. Sperm-mediated gene transfer have also been tried without convincing evidences(Nakanishi & Iritani, 1993). In chicken, one of favorite techniques is transferring a dozen of primordial germ cells(PGCs) transfected into an irradiated host embryo prior

to the microinjection of cells. In theory, it is very similar to embryonic stem(ES) cell injection into the developing mouse blastocyst cavity under a microscope. However, chicken embryo is very complex and unlike mouse embryo, we are only blindly injecting PGCs into the damaged embryos by irradiation. Therefore, subsequent development of the manipulated embryo with distinct prospective germ layer could be severly affected by the harsh injection pressure, physical damage and disturbance of cell layers. One of the ideal methodology is avoiding this harsh manipulation and cracking eggs which also reduce further development of the embryos to make transgenic chicken. After several year's of frustration(Lee et al., 1998; Park et al., 1994), we initiated such a gene transfer using transfected spermatozoa. To begin with, one should understand the mechanisms involved in early events of sperm-egg interactions to deliver relatively small number of DNA copies carried by a spermatozoon correctly. Second part of the review will deal with our preliminary data obtained from the electroporated spermatozoa for making transgenic chicken.

OVERVIEW ON EARLY FERTILIZATION EVENTS IN THE CHICKEN

It has clearly been known that reproductive tracts in hen contain sperm nests where ejaculated or inseminated spermatozoa are deposited for further use in fertilizing the ovulated ova. The deposited spermatozoa are released by various mechanisms as proposed by early investigators. Thus, inseminated hens can produce fertilized eggs for several days without a rapid decrease in fertility. Extensive studies have concentrated to elucidate the reproductive physiology underlying this events(Billard, 1998; Wishart and Staines, 1999), whereas early events occurring in penetrated eggs have been described only recently(Nakanishi et al., 1990; Perry, 1987). Polyspermy may occur in normal fertilization process, but only one spermatozoon develops into male pronucleus and unites with female pronucleus. Supernumerary spermatozoa penetrated into the ova undergo degeneration in the periphery of the central cytoplasm, thus being excluded from the zygotic formation during early cleavage. Further cleavage is so rapid that number of cells found in the oviposed eggs becomes around 8,000 to 60,000 cells during 26h-period after ovulation.

1. Sperm-egg interactions in the chicken

Extensive studies on fertilization in mammals have established cellular and molecular mechanisms occurring in early events of sperm-egg interactions. On the contrary, the fact that female gamete is comparatively huge to spermatozoa seem to be one of the reasons enough to discourage one who wish to study fertilization process in the chicken. The fertilization process discussed below may be important to apply sperm-mediated gene transfer technique because one can predict the fates of the foreign DNAs associated on and enclosed in the plasma membranes of spermatozoa through simple mixing and electroporation or liposomal transfection, respectively.

2. Sperm capacitation and acrosome reaction

It is not clear whether cock spermatozoa undergo a physiological modification called 'sperm capacitation' in the chicken. In mammals, spermatozoa are capacitated when they are exposed to the environment of female reproductive tracts. In chicken, spermatozoa deposited in the

Table 1. Studies on sperm acrosome reaction and sperm-egg fusion in the chicken[1]

Major findings	Systems used	Selected references
Sperm acrosome reaction	*In vivo* fertilization via EM	Okamura & Nishiyama(1978b)
Sperm IAM fusion with ova PM	*In vivo* fertilization via EM	Okamura & Nishiyama(1978a)
Phagocytic activity of ova PM	*In vivo* fertilization via EM	Koyanagi & Nishiyama(1980)

[1], EM, electron microscopy; IAM, inner acrosomal membrane and PM, plasma membrane.

sperm nests can fertilize the eggs for many days in a similar rate to freshly inseminated spermatozoa. Spermatozoa may be activated in the vicinity of the ovulated ova to fertilize it.

Acrosome in spermatozoa appears to be less dense and smaller than that of mammals. The ultrastructure of spermatozoa found in the vicinity of perivitelline layer(PVL) has been demonstrated(Table 1). The spermatozoa studied appear to be acrosome reacted when they penetrate the perivitelline layer. However, no other studies showing the fertilizing spermatozoa has been reported so far. It may be very difficult to look at all the egg surface to catch a penetrating spermatozoon in chicken egg. The acrosome reaction and penetration of spermatozoa into the egg have to be studied more extensively to demonstrate changes occurring in sperm surface membranes.

3. Sperm receptor activity of the PVL

As demonstrated in mammalian species, the solubilized zonae can bind to sperm surface and further prevent the treated sperm from binding to the intact unfertilized eggs. Similar sperm receptor activity was found in the extracellular matrix, the PVL of the chicken egg(Table 2). Although more studies have to be done to resolve whether the PVL components could induce acrosome reaction in spermatozoa, there are possibilities that incubation with isolated PVL suspension may cause any non-physiological changes in the active sperm plasma membrane. Only careful and elaborate experiments could resolve the sperm receptor activity of the PVL as demonstrated in mammalian species.

4. Sperm-egg perivitelline layer(PVL) interactions

According to the results obtained from *in vitro* and *in vivo* studies, sperm appears to make holes in the PVL by hydrolysing the extracellular matrix. The suggestion that sperm acrosin should be involved in this hydrolysis is not very convincing when compared to the evidences from mammalian species. The controvercy in number of bound sperm or sperm hydrolysis holes in the PVL is found ranging from 0 to 250,000 spermatozoa(Table 3).

The distribution of the bound spermatozoa or holes seems to be uneven or even over the PVL. PVL region overlying germinal disc showed 809

Table 2. Studies on sperm receptor activity on egg perivitelline layer(PVL) in the chicken[1]

Major findings	Systems used	Selected references
AR induction by PVL	Sperm-egg interactions *in vitro*	Koyanagi et al.(1988)
Reduction of sperm binding to PVL after PVL suspension incubation	Sperm-egg interactions *in vitro*	Howarth(1990)

[1], AR, acrosome reaction.

Table 3. Studies on sperm-egg perivitelline layer(PVL) in chicken

Major findings	Systems used	Selected references
Sperm hydrolysis holes in PVL	*In vitro* hydrolysis	Ho & Meizel(1975)
	In vitro sperm-egg interactions	Robertson et al.(1977)
	In vitro/*vivo* sperm-egg interaction	Robertson et al.(1998)
250,000 sperm found on PVL	Number of sperm-hydrolysis holes *in vivo*	Wisharts & Staines(1999)
Varying degree of holes present	Number of holes *in vivo*(0~100)	Staines(1998)
Uneven distribution of sperm on PVL (809 cells/Disc, 608 cells/Non-disc)	Sperm-PVL assay by Schiff's reagent	Bramwell & Howarth(1992)
25-fold sperm-hydrolysis holes in Disc region	Sperm-egg interactions *in vivo*	Wishart(1997)

holes, whereas other region had less holes, 608 holes(Table 3), indicating no significant difference between two regions. Other studies demonstrated that 25-folds sperm-hydrolysis holes were found in the disc area *in vivo* study. Judging from the summerized data presented in Table 3, it is probable that the bound spermatozoa or sperm-hydrolysis holes analysed by Schiff's reagent may cause this varying results among the experiments.

From the surveying the literature on the early fertilization events in the chicken, we can not rule out the other possible fertilization mechanism found in fish. In most fish, spermatozoa are guide by a fertilizing hole in the extracellular matrix, called micropyle. A fertilizing spermatozoon enters the micropyle to fuse with the plasma membrane of the oocyte in the overlying region of a very small cytoplasm. The micropyle is blocked by subsequently arriving spermatozoa(Joo et al., 1997). The other part of the chorion can still provide sperm recepting activity as in chicken.

Both in fish and chicken, they how enormous surface area to be tackled by active spermatozoa. There must be something in the egg surface to guide spermatozoa with a possible chemotactic activity or a physical guidance like the micropyle in the fish to target a very small area overlying small cytoplasm among the huge yolk area. If this speculation is right, then DNA-associated with spermatozoa may be easily carried into the oocyte as evidenced in fish.

TRANSGENESIS

1. Currently favored techniques

As in mammalian transgenesis, many ways of gene transfer have been tried with varying extents of results. These will not be dicussed in this review since this may be covered by other colleagues. However, I just summarized currently used techniques in Figure 1. All the techniques involve breaking eggshell to access the ova or embryos at different stages of development

2. Spermatozoa as DNA carrier : an alternative to transgenesis

In chicken if the sperm-mediated gene transfer is possible, it is very convenient to apply it without delay. Artificial insemination of cock spermatozoa is well established. There may be less intensive work to make transgenic chicken with the standard method(Table 4), we initiated

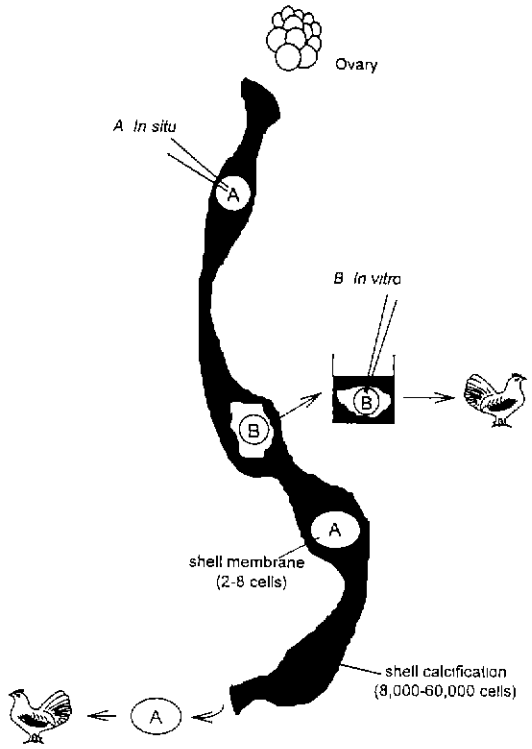

Figure 1. Different ways of cracking hen's egg to make transgenic chicken.

a study to establish the sperm-mediated gene transfer technique, already available in the laboratory for transgenic fish work.

The spermatozoa are very active after semen collection in chicken. It was important to maintain sperm motility and survived during transfection, otherwise the treated spermatozoa will not reach the sperm nests or ovulating ova. To establish optimum condition of sperm transfection with foreign DNA, various conditions of eletroporation were tested. Under the conditions used, no drastic decrease in the sperm survival was found as evidenced by the proportions of vitally-stained spermatozoa in the mitochondria when applied with Rh123 fluorescent dye(Table 5). The staining pattern of was found in the mitochondrial region of survived spermatozoa after DNA mixing and eletroporation, but not on the immotile or dead spermatozoa(Figure 2). Although the sperm motility decreased in the electroporated spermatozoa immediately after electroporating of spermatozoa in the presence of a foreign DNA, they survived for several hours on the bench. From this results, one of the major obstacles, decreased motility and survived was overcome.

The electroporated spermatozoa with the foreign DNA should contain the transferred DNA either associated on or enclosed in the sperm surface membrane. To test this, the transfected spermatozoa were analysed by polymerase chain reaction(PCR) with a pair of specific primers in the plasmid, pJJ9. As shown in Figure 3, both spermatozoa without and with pJJ9 mixing gave no PCR product after extensive centrifugal washings. DNase treatment did not affect the results. This results suggest that a simple and

Table 4. Studies on sperm-transfection with foreign DNAs in the chicken[1]

Major findings	Systems used	Selected references
[^{3}H]-DNA association with 19% sperm	DNA transfected sperm and HOP test	Trefil et al.(1992)
Dig-DNA association with 6~60% sperm	Incubation, transfection, electroporated sperm and AI	Nakanishi & Iritani(1993)
Increased survival of sperm after intravaginal AI	Lipofectin-treated sperm	Trefil et al.(1996)

[1], AI, artificial insemination and HOP, hamster ova penetration.

Table 5. Sperm viability as evaluated by Rh^{123} after electroporating spermatozoa in the presence of pJJ9 plasmid DNA under various conditions[1].

Electroporation condition (mA/pulse)	Total number of cells counted	Rh^{123}-positive cells(%)
Control	295	169(57.0)
0.02/3	286	164(57.0)
0.03/3	491	219(45.0)
0.05/3	504	268(53.0)
0.1/3	448	198(44.0)
0.1.5	415	176(42.0)

[1], Spermatozoa were stained for 5mun, washed twice, and prepared for fluorescence microscopy. Three independent experiments were pooled at each condition.

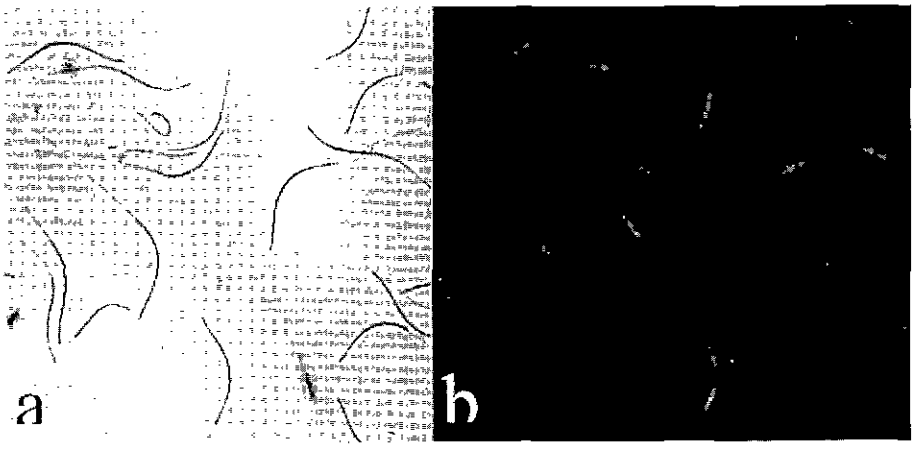

Figure 2. Vital staining of electroporated spermatozoa by Rh^{123}. Staining is found on the mitochodria of live spermatozoa (magnification is X 1000). Bright(a) and fluorescent field(b).

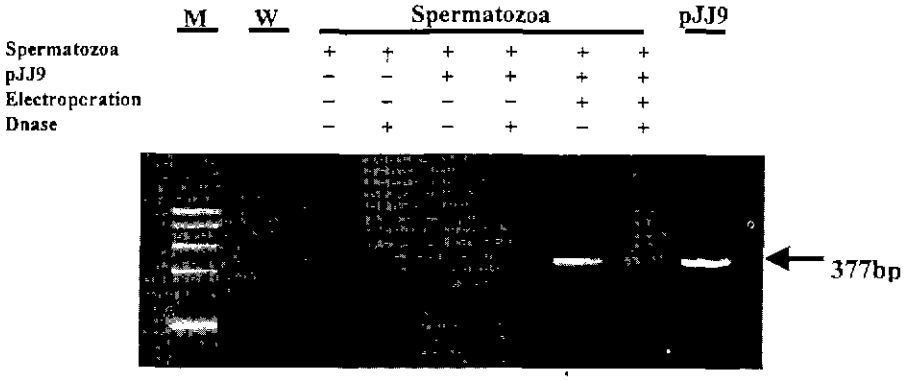

Figure 3. PCR detection of pJJ9 DNA fragment in electroporated spermatozoa in the presence of DNA. M, molecular weight standard; W, water alone and pJJ9, pJJ9 plasmid DNA alone.

brief incubation of spermatozoa and pJJ9 DNA should not be enough to carry DNA on the sperm surface membrane. Shearing force was also enough to remove all the loosely attached DNAs on the sperm surface by the centrifugal forces we used in this experiment. When the electroporated spermatozoa were analysed a specific PCR product, 377bp appeared and Dnase treatment before washing spermatozoa abolished the specific product. Instead a diffused and dim lane was found. This results indicated that the sperm-pJJ9 interaction may be much tighter than that of the mixed spermatozoa with pJJ9. The associated or enclosed DNAs can not be washed away by an identical centrifugal force and washing. However DNase treatment for 1h in a simple buffer may act on the DNA molecules enclosed in the cytoplasm already damaged spermatozoa in the milieu of simple buffer condition. Thus, the survived spermatozoa after electroporation indeed carry the foreign DNA within the cells.

3. Preliminary results from the transfected DNA into the fertilized eggs after AI

Immediately after the electroporated spermatozoa with pJJ9 DNA, the diluted semen was inseminated intravaginally for 4 successive days. Commercial eggs locally available and fertilized eggs inseminated with non-transfected spermatozoa(control) did not show any specific PCR product. Whereas pJJ9 DNA alone and fertilized eggs transfected with pJJ9 after AI gave a specific PCR product as mentioned earlier(Figure 4).These results demonstrate that the electroporated spermatozoa in the presence of a foreign DNA indeed carry the DNA, survived any physical manipulations(handling such as dilution, pipetting and AI), and retain sperm fertility and the foreign DNA together even in the chemical soup of female reproductive tracts.

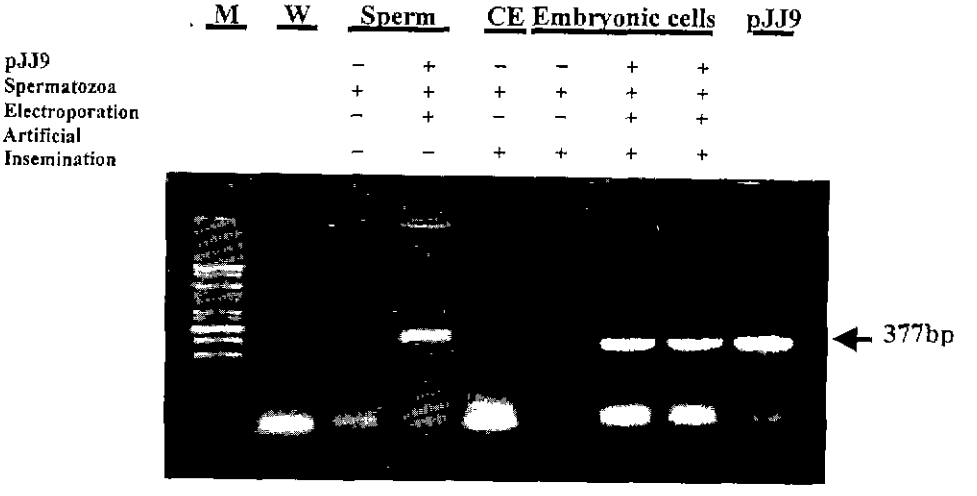

Figure 4. PCR detection of the transferred pJJ9 DNA fragment into the eggs following artificial insemination of electroporated spermatozoa. CE, commercial egg; M, molecular weight standard; W, water alone and pJJ9, pJJ9 plasmid DNA alone.

CONCLUSIONS

From these carefully designed experiments, we concluded that the electroporated spermatozoa could carry a foreign DNA and transfer into the ovulated ova and undergo fertilization and subsequent embryonic development. At present, we are following the next step forward to use practical genes containing endogenous chicken promoter(s) and human protein genes. The results obtained so far are promising with this alternative gene transfer. Sperm as DNA carrier could be used by avoiding any hindrance caused by other techniques, depending on rather *in vivo* process of fertilization after AI. Further understanding of early fertilization in detail will facilitate similar studies, and may find another way of cracking hen's eggs for transgenesis, without actually cracking them.

적 요

계란은 인간 및 다른 생물체로 부터 유래된 생물학적 활성 펩타이드 생산을 할 수 있는 가장 훌륭한 모델 중 하나임에도 지난 10여년간 상업적 가치를 지닌 유전자변환 닭을 생산하려는 노력은 기대에 미치지 못하고 있다. 이는 비교적 큰 난황 물질로 인하여 난자조작이 용이하지 않은 것이 가장 큰 장애물이 되고 있기 때문이다. 현재까지 주로 많이 이용되고 있는 방법이외에 정자를 매개체로 하여 외래 유전자를 수정시 도입하고자 하는 것은 어류와 같은 동물에서는 성공하고 있으며 이는 그 동안의 이들 동물에서 수정 과정 등이 비교적 상세히 알려져 있는 것이 큰 도움이 되었다. 본 토의는 이와 유사한 방법을 위한 단계로 닭의 수정 연구에 의해 축적된 닭의 초기수정 기작이 유전자변환 닭의 생산에 이용될 수 있는 방안에 대하여 논의하고자 한다.

(주제어 : 초기수정, 정자내 외래 DNA 주입, 유전자 변환 닭 생식).

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