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Production of Transgenic Chicken by Using Embryo Culture Techniques

I. S. Jeon, D. H. Yoon, E. W. Park, W. G. Nho, C. H. Choi, J. H. Lee, H. H. Lee, I. C. Cheong and J. Y. Han[1]

National Livestock Research Institute, RDA, Korea.

[1] School of Agricultural Biotechnology, Seoul National University, Korea

수정란 배양 기술을 이용한 형질전환 닭 생산

전익수 · 윤두학 · 박응우 · 노환국 · 최철환 · 이지현 · 이향흔 · 정일정 · 한재용[1]

농촌진흥청 축산기술연구소, [1]서울대학교 농생명공학부

ABSTRACT : The goal of this paper was to examine the quality zygote-acquiring method for *in-vitro* culture and the in-vitro culture method of the acquired zygote from a technological perspective. We have reported the results on the introduction of foreign DNAs using the described culturing method. After performing *in-vitro* and surrogate eggshell culture on a zygote acquired from the abdomen of a hen, 25.8% hatchability was acquired. After microin-jecting foreign DNAs into the acquired zygote and performing *in-vitro* and surrogate eggshell culture using the same method, 13.1~11.7% hatchability was acquired. Having compared the developments of the control subjects and the experimental subjects, the viability of the experimental subjects on the 4~5th day of culturing was much lower compared to that of the control subjects. This is a result that shows that the microinjection process of foreign DNAs might have a negative effect on the existence of the embryo; therefore, various technical attempts should be made to minimize such negative effects. Having microinjected foreign DNAs into the zygote of a hen to produce transgenic chickens, 3 transgenic founders were produced and 70 G1 progeny were produced as a result of the progeny test that had been performed to the present.

(Key words: chicken, zygote, *in vitro* culture, surrogate eggshell culture, transgenic, DNA microinjection)

INTRODUCTION

For reasons of embryological characteristics, most of the ontogenesis processes in birds, excluding fertil-ization and early embryo development, proceed inside the eggshell (Rowlett and Simkiss, 1987; First and Haseltine, 1991; Naito, 1991). Therefore, when applying biotechnological techniques to the zygote, it should be equipped with an established artificial *in-vitro* culture system for the ontogenesis of birds, while, for mammals the biotechnological-treated zygote must be transplanted into the maternal part after in-vitro culture for early development. In addi-tion, since the ovum of birds has much more yolk than the oocyte of mammals, it is large in size and can be broken easily. The ovum of a chicken is fertilized after being penetrated by sperm about 15 minutes after ovulation at the infundibulum (Olsen and Neher, 1948). The zygote begins cleavage with a narrow cavity in between after dividing itself into two. The first cleav-age starts when the zygote is at the isthmus and then the zygote divides itself into the 256-cell stage when it reaches the uterus. Then, it divides into an epiblast and a hypoblast layer and forms a subgerminal cavity between them just before oviposition. Oviposition starts around the time when it forms the hypoblast layer and the number of cells increases to about 60,000 (Spratt and Haas, 1961; Patten, 1971; Eyal-

Giladi and Kochav, 1976; McMaster and Modak, 1977; Kochav et al., 1980; Eyal-Giladi, 1984; Perry, 1887; Rowlett and Simkiss, 1987; Naito et al., 1990; Naito et al., 1991). After ovipositioning occurs, the fertilized egg hatches at an adequate temperature and humidity for embryo development in a rocking incubator.

Meanwhile, in the process of egg formation, as stated earlier, if the ovum that has a lot of yolk ovulates to the infundibulum and meets the sperm, it continues to the egg stage as a fertilized egg. If it does not meet the sperm, it continues to become an unfertilized egg. If the ovulated ovum passes the infundibulum and reaches the magnum, thick albumin surrounds the ovum. When it reaches the isthmus, an eggshell membrane is formed and thin albumin is deposited. After that, the ovum that has passed the isthmus stays in the uterus at that point for over 20 hours and then oviposition is achieved after the calcification process (Crooks and Simkiss, 1975; Dunn et al., 1981; Solomon, 1991). Hence, in order to produce a transgenic chicken by microinjection of foreign DNA into the zygote, the zygote must be taken out before the first cleavage is done inside the oviduct of a hen. After the foreign DNA is microinjected into the zygote, the zygote should be transplanted into the oviduct of a hen, but currently no such technology exists to accomplish this. Therefore, as stated earlier, an artificial *in-vitro* culture system must be established for embryo development and for the further ontogenesis within the oviduct of the maternal body. Early research on artificial *in-vitro* cultures was performed using culture dishes and plastic wraps for notification of the early stage embryos. However, with such a method, the inadequate shape of the culture vessel physically limited the formation of the extra-embryonic membrane. Also, since 75~80% of the calcium supplied to the embryo during development is supplied through the chorioallantoic membrane of the eggshell, if artificial *in-vitro* culture is performed on the embryo without an eggshell, then there is a lack of calcium supply to the embryo and therefore calcification of the bone is hindered. For such reasons, hatching would be difficult (Auerbach et al., 1974; Crooks and Simkiss, 1974, 1975; Dunn and Boone, 1976; Dunn and Fitzharris, 1979; Dunn et al., 1981; Tuan, 1980; Ono and wakasugi, 1983; Rowlett and Simkiss, 1987; Solomon, 1991; Elaroussi et al., 1994). This has led to the development of a surrogate eggshell culture system in which the eggshell of a bird is used as a culture vessel for artificial *in-vitro* culture (Ono and Wakasugi, 1984; Rowlett and Simkiss, 1987; Perry, 1988; Ono et al., 1994a). As for the surrogate eggshell, in order to culture the embryo of a chicken, the eggshells of turkeys and chickens are used. Through this surrogate eggshell culture system, the embryo development of almost all stages, except the embryo development that was processed inside the oviduct of the maternal body, was possible with the use of an artificial *in-vitro* culture (Ono and wakasugi, 1983; Rowlett and Simkiss, 1987). After that, the Perry (1988) Group of the Roslin Institute of Scotland succeeded for the first time in extracting the 1-cell stage embryos and hatching a chick through a perfect artificial *in-vitro* culture method. That is to say, all the development processes, including the embryo development that had proceeded only inside the oviduct of the maternal body of the hen, was performed perfectly. This technology is being used widely as a basic culturing method for diverse embryo development research including the production of transgenic chickens and chimeras. In addition, with the improvement in the interval and angle of rocking, the adequate size of the surrogate eggshell to be used and the treatment process and the ingredients of the culture medium to be used, the chances of hatching have been improved rapidly compared to earlier years (Naito et al., 1990; Perry and Mather, 1991; Naito et al., 1991; Love et al., 1994; Naito et al., 1994; Ono et al., 1994b; Ono et al., 1995; Jeon et al., 1997; Sherman et al., 1998; Jeon et al., 1998; Sang, 2000; Jeon, 2000). Therefore, in this study, we will examine the zygote acquisition method of good quality for the *in-vitro* culture of the chicken zygote and the *in-vitro* culture method of the acquired zygote from a technological viewpoint; and further, to report on the series of results that introduced foreign DNA using the above culturing method.

PREPARATION OF THE DONOR HEN

To extract the zygote of the chicken before its initial cleavage, the donor hen must be prepared with careful selection. The state of the donor hen is closely related to the quality of the zygote. If there is a possibility of successive ovipositioning in a hen today and tomorrow, the ovum to be ovipositioned tomorrow will be ovulated to the infundibulum 2 hours after today's oviposition. About 15~30 minutes after that, the zygote passes the oviduct magnum and at the same time, thick albumin is secreted and wraps around the zygote, making it look like an elastic capsule. When the zygote surrounded with thick albumin reaches the isthmus, the initial cleavage begins and the 4~8th cell stages are completed and the eggshell membrane begins to be formed along with that. Therefore, in order to acquire the zygote, the zygote must be extracted by slaughtering the donor hen when the zygote has not yet passed the isthmus and stays at the magnum(Fig. 1). In addition, by the nature of the experiment, slaughtering one donor hen to acquire one zygote is more effective and economical than acquiring the zygote by anethetizing the hen and performing surgery for the purpose of reuse. The donor hen must be decided on by referring to the oviposition record before slaughtering. This is because there is no need to kill a hen that is not expected to have an oviposition tomorrow calculated by the reference of the clutch.

Fig. 1. The donor hen to supply the zygote will be slaughtered 2 hours and 15 minutes after oviposition on the experiment day. The surgeon opens the abdomen of the donor hen and extracts the oviduct containing the zygote(arrow) and passes it on to the culture room for culturing.

PREPARATION OF THE SURROGATE EGGSHELL

The zygote acquired from the donor hen for the purpose of microinjection of the foreign DNA was extracted from the body 22 hours before the normal eggshell was formed. Therefore, the extracted zygote needs a culture vessel for embryo development and requires an environment similar to that of the inside of the oviduct of the hen for normal development. A glass vessel or the eggshell of a chicken is mainly used as a culture vessel. When the glass vessel is used, it is only used for about 24 hours when the zygote in its natural state develops within the oviduct of the hen(Perry, 1988; Naito et al., 1990). It must then be transferred to the surrogate eggshell. In order to eliminate changing the process in the culture vessel, it is more effective to use the surrogate eggshell in the first place. For the first 4 days, the surrogate eggshell should be an eggshell that has the same weight as the preceding egg that was ovipositioned before the hen that supplied the zygote was slaughtered. Then, after 4 days have passed, the eggshell that is 25g heavier than the preceding egg should be used. That is to say, the weight of the preceding egg that was ovipositioned before the hen that supplied zygote was slaughtered is used as a standard for selecting the surrogate eggshell. However, this cannot be seen as a perfect method because the weight of the egg ovipositioned today and the weight of the egg that will be ovipositioned tomorrow do not match exactly.

CULTURING THE ZYGOTE

As stated above, a fresh egg that has the same weight as the ovipositioned egg of the donor hen before it was slaughtered is used in the culture vessel

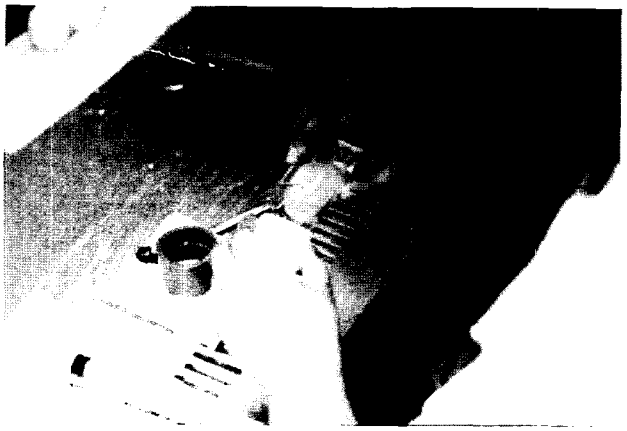

Fig. 2. The zygote with yolk and thick albumen capsule was transferred to a surrogate eggshell as a culture vessel.

for the *in-vitro* culture of the chicken zygote. A diameter of 3.5 cm of the sharp end of the selected fresh egg is cut off using a dental drill, and then the contents are thrown away and examined for any damaged parts. The zygote extracted from the donor hen is transferred to the prepared surrogate eggshell. The transfer method is; first, the oviduct is peeled carefully so that it cannot damage the thick albumin covering with the zygote. Then, the zygote is transferred to the surrogate eggshell using a 50ml beaker (Fig. 2). After the transfer, 5ml of thin albumin that has been adjusted to pH 7.30~7.35 by CO_2 gas is added. Then the entrance of the surrogate eggshell is covered with a plastic dish and a primary culturing is performed for one day in an humidity-saturated incubator at 41.5℃ with 5% CO_2. For the culture medium of secondary culturing, thin albumin is acquired from the fresh egg one day before culturing and is used after being heated to 37.6℃. For the secondary culturing, prepared culture medium is poured to the fullest into the surrogate eggshell and then sealed with a wrap. The amount of the filled culture medium can differ according to the size and the content capacity of the surrogate eggshell, but it is about 20~25ml per one. After completely filling with the culture medium, secondary culturing is performed for 3 days in a 37.6℃ and 65~70% humidity incubator vessel, and by rocking it 90° every 11 minutes. After 4 days of culturing using the above method, the culture vessel is replaced once more for a 3rd culturing. The preparation of the surrogate eggshell for the 3rd culturing involves selecting a fresh egg that is about 25g heavier than the preceding egg that was ovipositioned before the donor hen was slaughtered. Then, the blunt end is cut off at a 4cm diameter and the contents are thrown away and examined for damaged parts. The embryo and the contents that have been cultured for 4 days are transferred to the 3rd culturing surrogate eggshell. The embryo and the contents are poured into a glass vessel with a diameter of 15 cm after covering the vessel with a kitchen wrap. Then the contents are wrapped with the wrap, pushed into the prepared surrogate eggshell slowly and the wrap is pulled out carefully. Then the entrance of the surrogate eggshell is sealed with the wrap and cultured for 15 days in a incubator at 37.6℃ and 65~70% humidity, while rocking it 30° every 30 minutes (Perry, 1988; Perry and Mather, 1991; Jeon, 2000). The rocking is stopped 1~2 days before hatching. When the beak of the chick inside the surrogate eggshell breaks the chorioallantoic membrane, then 1 or 2 holes must be made on the covered wrap with a 26G injection needle to help the chicks breathe (Rowlett and Simkiss, 1987; Perry, 1988; Naito et al., 1990). When the chicks begin to chirp, the wrap is removed and a plastic dish is used to cover the entrance. When the blood in the blood vessel of the chorioallantoic membrane is completely absorbed into the body of the chick and cannot be seen, then the chorioallantoic membrane should be carefully pushed to the far side of the eggshell using sterilized tweezers. This is to prevent the chorioallantoic membrane from sticking onto the body of the embryo so that it will not hinder the breathing and movement of the chicks. The success of the hatching should be checked the next day.

INJECTION OF FOREIGN DNA

The objective of extracting the zygote from the abdomen of the hen is to produce transgenic chickens through microinjections of foreign DNAs into the zygotes. For the DNA, the onset-control part of the

β-galacosidase gene (lac Z) of the pCMV β vector was separated and retrieved to be used after cutting(Love et al., 1994; Hong et al., 1998). Injection density was 25 μg/ml and the injection amount was 2 nl. For the injection pipette to inject the DNA into the zygote, a micropipette(GD-1, Narishige Co., Japan) was processed with a micropipette puller(PC-10, Narishige Co., Japan) and micropipette grinder(EG-40, Narishige Co., Japan) so that it would have a 60 μm in outer diameter and 30° angle. Then, it was sterilized at 110℃ for 6 hours. For micromanipulation of the DNA injection, we took off the upper arm part of the mechanical stage of a general optical microscope and attached a manipulator(MN-151, Narishige Co., Japan) to it as shown in Fig. 3 and used a 10 ml glass injector for injection. To secure a view of the microinjection area, we attached a stereoscopic microscope (SZH-10, Olympus Co., Japan) to a universal stand (SZ-STU2, Olympus Co., Japan) at an angle of about 30° perpendicular so that the germinal disc inside the surrogate eggshell could be seen (Fig. 3). To inject the foreign DNA into the germinal disc of the zygote, as shown in Fig. 3, the zygote was put on the mechanical stage and then the micropipette was moved so that it was right above the germinal disc. Then, the mechanical stage was moved up for injection using the adjustment knob. When the micropipette was

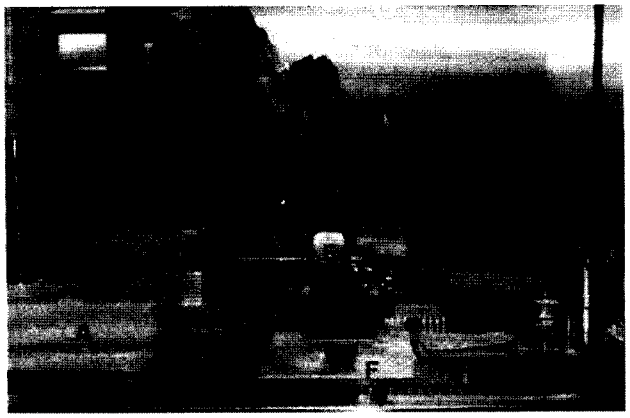

Fig. 3. Microinjection apparatus and DNA injection. A, universal stand; B, fiber light system; C, dissecting microscope; D, micromanipulator; E, surrogate egg-shell; F, transformed microscope; G, injector. The mechanical stage was moved up for injection using the microscope's adjusting knob.

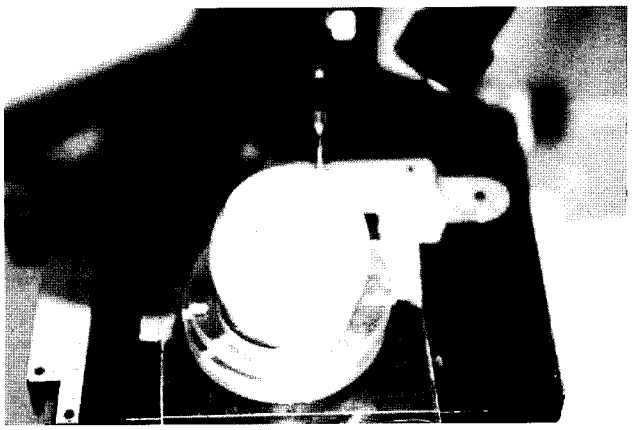

Fig. 4. The transgene construct in a volume of 2nl was injected into the center of the germinal disc.

inserted into the germinal disc to about 100μm in depth, 2nl of the prepared gene were microinjected using an injector.

SCREENING FOR TRANSGENIC BIRDS

After the DNA was injected into the zygote, a PCR analysis was performed to examine the insertion of the foreign DNA that was injected from the chorioallantoic membrane, blood, and germ cells of all chicks that were hatched by *in-vitro* and surrogate eggshell culture. For the analysis of germ cells, when the hatched chick reached puberty, if it was male the sperm was used for analysis, but in case of a female a fertilized egg was produced and incubated for 3 days and analysis was performed on the embryo. A transgenic founder that was judged to be positive from the germ cells was artificially inseminated for production of G1 progeny and the produced G1 progeny was examined for the insertion of the foreign DNA that was injected from the chorioallantoic membrane and blood, through PCR analysis.

VIABILITY AND HATCHABILITY OF CULTURED EMBRYOS

The results of *in-vitro* and surrogate eggshell cul-

Table 1. Viability of embryos from culture control and injection with DNA

Embryos	Birds	Zygotes(n)	Survival of embryos during culture			No. of hatched	No. of I week
			4days(%)	11days(%)	19days(%)	chicks(%)	old birds(%)
Culture control	CL*	120	103(85.8)[a]	60(50.0)[a]	55(45.8)[a]	31(25.8)[a]	26(21.7)[a]
DNA injection	CL*	176	115(65.3)[b]	46(26.1)[b]	40(22.7)[b]	23(13.1)[b]	19(10.8)[b]
	KNC**	248	146(58.9)[b]	65(26.2)[b]	48(19.4)[b]	29(11.7)[b]	19(7.7)[b]

*: Commercial layer.

**: Korean Native Chicken.

[a,b]: Mean within a column with different superscripts are significantly different($P<0.05$).

ture after injection of foreign DNAs into the germinal disc of the zygote are shown in Table 1.

When the zygote of the commercial layer was injected with a DNA, the viability was 65.3% until the 4th day of culturing, 26.1% until the 11th day of culturing, and 22.7% until the 19th day of culturing. The hatchability was 13.1% and the early viability of the hatched chick for 1 week was 10.8%. When the zygote of a Korean Native Chicken (KNC) was injected with a DNA, the viability was 58.9% until the 4th day of culturing, 26.2% until the 11th day of culturing, and 19.4% until the 19th day of culturing. Hatchability was 11.7% and the early viability of the hatched chick for 1 week was 7.7%. The results were no significantly different between commercial layer and Korean Native Chicken($p<0.05$). The viability of the control subject that had omitted the DNA injection process was 85.8% until the 4th day of culturing, 50.0% until the 11th day of culturing, and 45.8% until the 19th day of culturing. The hatchability was 25.8%, and the early viability of a hatched chick for 1 week was 21.7%. The viability, hatchability and the early viability of the hatched chick for 1 week of the control subject that did not have a DNA injected and had only artificial *in-vitro* and surrogate eggshell culturing performed, was significantly higher than the experimental subject in which foreign DNAs were microinjected ($p<0.05$). There was a radical decrease in viability on the 4~5th and 21st culturing day on the whole for the viability pattern of the control subject and the experimental subject. Especially, in the case of the experimental subject, the viability showed a larger drop than the control subject showed on the 4~5th culturing day (Fig. 5). This is a result that shows that the microinjection process of the foreign DNA might have a negative effect on the survival of the embryo and various technical attempts seem necessary to minimize such negative effects.

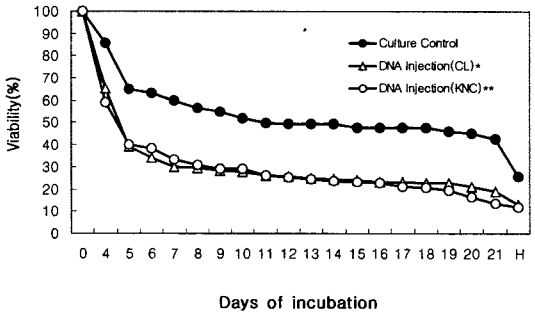

Fig. 5. The viability patterns of chick embryos from culture control and DNA injection.

Table 2. Transgenic chicks by DNA injectionPCR positive

Bird	Zygote injection(n)	No. of hatched chicks(%)	PCR positive semen (male)	PCR positive 3day embryo(female)
CL*	76	23(13.1)	1	0
KNC**	248	29(11.7)	1	1

* : Commercial layer.

** : Korean Native Chicken.

Fig. 6. PCR analysis of DNA from semen and 3-day-embryo.

Table 3. Progeny test of transgenic founder by PCR*

Positive chicken ID number	No. of chicks hatched	No. of positive chicks(%)
1010(♀)	285	70(24.6)

* Test for transgene in DNA chorioallantoic membrane (CAM) and blood from chicks

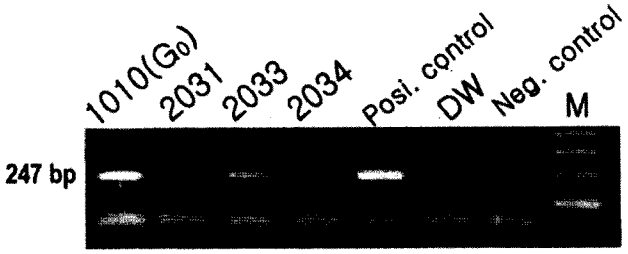

Fig. 7. PCR analysis of CAM and blood from G1 transgenic chicks(G1 ID No. : 2031, 2033, 2034).

PRODUCTION OF TRANSGENIC CHICKENS

Having micro-injected foreign DNAs into the zygote of chickens to produce transgenic chickens, 29 of the 248 zygotes of KNC were hatched. After examining the foreign DNA injection through a PCR analysis using germ cells, one male and one female were found, respectively. In the case of the commercial layer, 23 of the 176 zygotes were hatched and there was one male positive germ cell after puberty. To the present and according to the progeny test that had been performed, 285 G1 progeny were produced; and, after having analyzed the blood of the chorioallantoic membrane, 70 of the G1 progeny were judged to be positive.

CONCLUSIONS

As a result of this research, the DNA microinjection technique that has been combined with the *in-vitro* and surrogate eggshell culture techniques for the chicken zygote will maximize the use of useful human genes that will be available in the future. Also, it can be used as a core technology for the production of transgenic chickens that secrete useful proteins into the egg albumin.

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