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Comparison of Viable Rates of Chick Embryos by Different Eggshell Window Positioning

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닭 배자 조작을 위한 난각 주입부위별 생존율 비교

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ABSTRACT

This study was conducted to compare the survival rate of chick embryos among different eggshell window positions and to search for the most appropriate injection position. The eggshells were punctured at blunt-end, sharp-end and side-up with a sterilized fine forceps, respectively. The survival rate of sharp-end window was higher than the other window positions. Injection of Dulbecco's modified eagle's medium (DMEM) through blunt-end window (BE1) was impossible because inner cell membrane was obscure. The 2 μL DMEM was injected into 2.5 d-old embryo blood vessel through sharp end window. To prevent hemorrhages at the point of injection, the air bubbles were injected into the embryo blood vessel. The survival rate of chicks embryo in sharp end window was about 17.0%. Therefore, this sharp-end window system will be helpful for the production of germline chimera or transgenic chicken using primordial germ cells (PGCs).

(Key words: chicks embryo, viable rate, window positioning)

INTRODUCTION

Since Waddington (1932) reported that embryos which were isolated from 1-day incubated egg and then explanted onto solid medium continued to develop for about 2 days, a number of attempts have been made to produce transgenic chicken by the use of *in vitro* culture system (Perry, 1988; Ono et al., 1994; Naito et al., 1995). But this *in vitro* culture system was a time-consuming process with considerable morbidity. As the primordial germ cells were isolated from embryos (germinal crescent and blood vessel) and injected into this region successfully, the *in vitro* culture system is not the best way to produce germline chimeric chicken. And this *in vitro* culture system is not appropriate to produce germline chimera chickens using primordial germ cells as a vector.

The early stages of chick development are relatively inaccessible, as they take place within

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oviduct of the hen. The first cell division takes place at the isthmus of the oviduct, within 4 to 5h after fertilization of the ovum. Subsequently, cell divisions, which occur in the uterus while the shell membrane is laid down and calcification of the shell takes place, are very rapid. By the time the egg is laid, the embryo consists of at least 60,000 cells (Foulkes, 1990).

To investigate the possibilities of poultry genetic manipulation by introducing cloned DNA into the single cell embryo, many researchers reported that transgenic chicks could be produced *in vitro* embryo culture (Perry, 1988; Dugan et al., 1991; Ono et al., 1994). Perry (1988) devised a complete culture method for hatching chick embryos from single-cell stage obtained from the posterior portion of the magnum. It was consisted of three culture systems, using surrogate eggshell method for the final phase of the embryo culture. The embryos used in Perry's (1988) culture method were surrounded by a certain amount of dense albumen capsule around the yolk. However, Naito et al. (1995) developed a novel culture system for chick embryos obtained from anterior portion of the magnum in which only a thin layer of dense capsule was formed around the yolk. *In vitro* culture system for fertilized chick ova is useful for analysing the developmental processes of embryos and for the production of transgenic chickens. But these methods have some disadvantages: difficulties of manipulation *in vitro*, and low survivability with cost- and time-consuming efforts.

Recently, the production of transgenic chicken has been attempted by microinjection of primordial germ cells, which were transferred recombinant DNA into germinal crescent (stage 6 to 8) and blood vessels (stage 19 to 20) through the window (Han et al., 1994). The purpose of this study was to establish the fundamental data for the production of germline chimera chicken. Thus this study was conducted to compare the hatchability on window position, and to determine the optimal egg window position for microinjection into the embryonic blood vessel.

MATERIALS AND METHODS

1. Fertilized egg preparation

Fertilized eggs were obtained from Single Comb White Leghorn (WL) which were maintained at Experimental Animal Farm, Seoul National University. Artificial insemination was performed twice a week with semen from WL males. Fertilized eggs were prewarmed overnight at room temperature. Before the incubation, egg shell was wiped with 70% ethanol and incubated at 37.5℃ with 60 to 70% relative humidity and every 2hrs rotating at 90 degree.

2. Opening egg shell

To compare the chicks embryo viability among egg window positions, three kinds of treatments (blunt-end, side-up and sharp-end) and control (1), (2) were used in this experiment (Figure 1). After the fertilized eggs were incubated for 24h, the eggs were transferred and incubated at 37.5℃ with 60 to 70% relative humidity in unrotating incubator. After 12h incubation, the egg shell was cleaned with 70% ethanol and punctured about 5 to 10 mm diameter with sterilized fine forceps. The incubation procedure between windowed eggs and controls of this experiments were shown in Figure 2. Before egg-shell was punctured, eggs were incubated for 48 h at 37℃ in rotating incubator and maintained in unrotating incubator for 12h. After these incubation procedures, the egg-shell

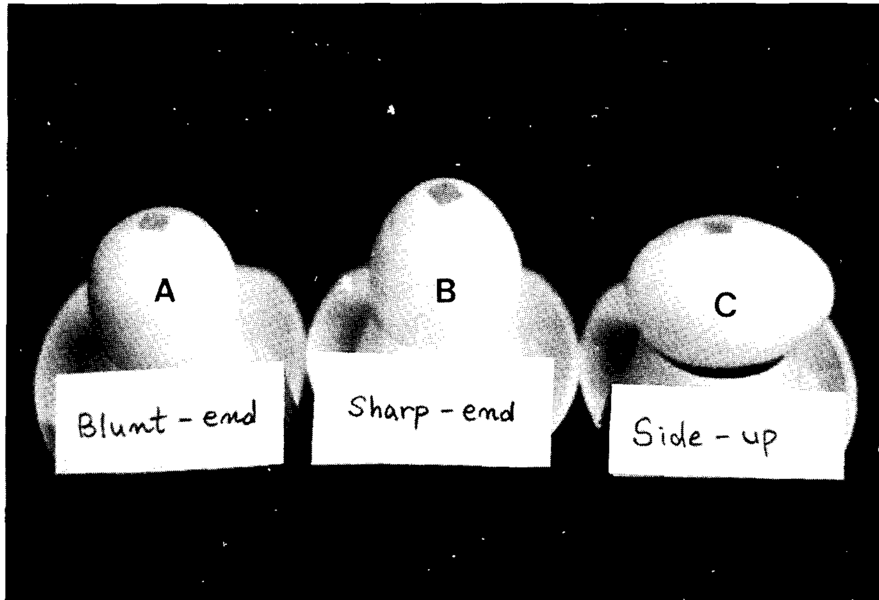

Figure 1. Windowed egges. (A) Blunit-end, (B) Sharp-end, (C) Side-up.

Window Position	Experimental Procedure							
Blunt-end	Rotating[1) (48hrs)	Unrotating[1) (12hrs)	Puncture[1) (2min)	Injection[1) (2min)	Sealing[1) (1min)	Unrotating[1) (12hrs)	Rotating[1) (16days)	Hatching (2days)
Side-up	Rotating[1) (48hrs)	Unrotating[2) (12hrs)	Puncture[2) (2min)	Injection[2) (2min)	Sealing[2) (1min)	Unrotating[2) (12hrs)	Rotating[1) (16days)	Hatching (2days)
Sharp-end	Rotating[1) (48hrs)	Unrotating[3) (12hrs)	Puncture[3) (2min)	Injection[3) (2min)	Sealing[3) (1min)	Unrotating[3) (12hrs)	Rotating[1) (16days)	Hatching (2days)
Control(1)	Rotating[1) (48hrs)	Unrotating[1) (12hrs)	Puncture[1) (2min)	Injection[1) (2min)	Sealing[1) (1min)	Unrotating[1) (12hrs)	Rotating[1) (16days)	Hatching (2days)
Control(2)	Incubated at 37℃, humidified, rocked (90 degree) incubator							

(shaded box) : Incubated at 37℃, humidified incubator

(blank box) : at room temperature

[1)]: Incubated blunt-end, [2)]: Incubated side-up, [3)]: Incubated sharp-end

Figure 2. The incubation procedures of windowed eggs and controls.

was punctured within 2 min and stayed at room temperature for more 2 min for the injection time and then sealed with paraffin film within 1 min (Whatman Co.). The sealed eggs were

incubated in unrotated-incubator that can safely develop chicks embryo. Finally, sealed eggs were incubated at 37℃, rotating, humidified incubator. Candling was performed at 5 day, 12 day, 19 day of incubation, respectively.

3. Injection of DMEM into chicks embryo

To determine the hatchability of eggs injected through the sharp-end window, a volume of 2 μL DMEM media (Sigma Co.) without serum was injected into a blood vessel with a specially designed 100 μL micro-glass pipette (Drummond Scientific Co., USA) under stereomicroscope. Micropipette, inner surface coated with solution of silicon (Sigmacote®, Sigma Co., USA) was used for injection. To prevent hemorrhages from the point of injection, air bubbles were injected into the embryo blood vessel.

After DMEM was injected into blood vessel of chicks embryo, the window was sealed with two folds of paraffin film (Whatman Co.). Those eggs were further incubated with 37℃ in unrotating incubator for 12 h. And then the eggs were transferred and incubated in rotating incubator that was rotating every 2 h at 90 degree.

RESULTS

The embryo viability of chicks was shown in Table 1 to 3. The survival rate of chicks embryo for the blunt-end (BE) window eggs in Table 1. BE was devided into two groups (BE1 and BE2) if it contains egg inner shell membrane of chicks embryo at blunt-end. BE1 had its own inner

Table 1. Survival rate of the blunt-end windowed eggs

Treatment	Experiment	Egg number	Survival number (%)			Dead embryo number	Hatched number
			5 days	12 days	19 days		
1	1	17	14 (95.0)	13 (90.0)	12 (86.3)	2 (11.8)	10 (58.8)
	2	30	30 (100.0)	28 (93.3)	26 (86.7)	6 (20.0)	20 (66.7)
	3	33	32 (97.0)	31 (94.0)	31 (94.0)	4 (12.1)	27 (81.8)
Total	3	80	76 (95.0)	72 (90.0)	69 (86.3)	12 (15.0)	57 (71.3)
2	1	17	14 (82.4)	6 (35.3)	3 (17.4)	2 (11.8)	1 (5.9)
	2	36	22 (61.1)	10 (27.8)	8 (22.2)	6 (16.7)	2 (5.6)
	3	36	34 (94.4)	8 (22.2)	6 (16.7)	5 (13.9)	1 (2.8)
Total	3	89	70 (78.7)	24 (27.0)	17 (19.1)	13 (14.6)	4 (4.5)

Treatment 1 : Inner shell membrane was not removed from embryos.
Treatment 2 : Inner shell membrane was removed from embryos.
( ) : Percentage of survived embryos.

Table 2. Survival rate of the sharp-end windowed eggs

Experi-ment	Egg number	Survival number (%)			Dead embryo number	Hatched number
		5 days	12 days	19 days		
1	87	81 (93.1)	65 (74.7)	64 (73.6)	36 (41.4)	28 (32.2)
2	66	64 (97.0)	55 (83.3)	47 (71.2)	19 (28.8)	28 (42.4)
3	50	45 (90.0)	40 (80.0)	38 (76.0)	15 (30.0)	23 (46.0)
Total 3	203	190 (93.6)	160 (78.8)	149 (73.4)	70 (34.5)	79 (38.9)

( ) : Percentage of survived embryos.

Table 3. Survival rate of the side-up windowed eggs

Expeiment	Egg number	Survival number (%)			Dead embryo number	Hatched number
		5 days	12 days	19 days		
1	110	81 (73.6)	75 (68.2)	71 (64.5)	28 (25.5)	43 (39.1)
2	64	45 (70.3)	39 (60.9)	34 (53.1)	11 (17.2)	23 (35.9)
3	50	39 (78.0)	30 (60.0)	23 (46.0)	5 (10.0)	18 (36.0)
Total 3	224	165 (73.7)	144 (64.3)	128 (57.1)	44 (19.6)	84 (37.5)

( ) : Percentage of survived embryos.

shell membrane. BE2 means that inner shell membrane was removed at time of DMEM injection. The hatchability of BE1 and BE2 was 71.3% and 66.8%. The survival rates of sharp-end windowed eggs at 5 and 19 day of incubation (Table 1) were higher than those of side-up windowed eggs. During day 5 to day 19 of incubation (Table 2 to 3), the survival rate of sharp-end windowed eggs was higher than side-up windowed eggs. There's no significant difference in hatchabilities between eggshell window positions. The average hatchabilities of side-up and sharp-end windowed eggs were 37.5% and 38.9%, respectively. Table 4 shows the embryo survival rate for the non-windowed egg control. The control (1) was incubated in the same steps as the three kinds of window position. And the control (2) was incubated at 37℃, humidified incubator which was rotated about 90 degree every 2 hours. The proportion (%) of dead embryo in control (1) was higher than that in control (2).

According to the results of hatchabilities for window position, the survival rate of blunt-end (BE1) windowed eggs was higher than that of sharp-end windowed eggs but DMEM was

Table 4. Survival rate for control eggs

Expeiment	Egg number	Survival number (%)			Dead embryo number	Hatched number
		5 days	12 days	19 days		
Control(1)[1]	93	89 (95.7)	86 (92.5)	83 (89.2)	14 (15.1)	69 (74.2)
Control(1)[2]	81	81 (100.0)	77 (95.1)	76 (93.8)	7 (8.6)	69 (85.2)

( ) : Percentage of survived embryos.

[1] Control(1) : incubated in the same steps as the three kinds of window position.

[2] Control(2) : incubated in the same condition and not punctured.

Table 5. Survival rate after the injection into the embryo blood vessels through sharp-end windowed egg

Expeiment	Egg number	Survival number (%)			Dead embryo number	Hatched number
		5 days	12 days	19 days		
1	36	26 (72.2)	17 (47.2)	14 (38.9)	9 (25.0)	5 (13.9)
2	35	28 (80.0)	18 (51.4)	16 (45.7)	10 (28.6)	6 (17.1)
3	35	28 (80.0)	22 (62.9)	16 (45.7)	9 (25.7)	7 (20.0)
Total (77.4)	106 (53.8)	82 (43.4)	57 (26.4)	46 (17.0)	28	18

( ) : Percentage of survived embryos.

injected through sharp-end windowed egg to measure the hatchability of injected eggs because inner shell membrane was obsecure and it is impossible to inject DMEM through the blunt-end window (Table 5). Mortality of blunt-end window eggs with injection was high compared to only sharp-end windowed eggs at early developmental stage. As a result, hatchability was decreased to 17%.

As the egg window positions, survival rate of chicks embryo was shown in Figure 3. The results of this experiment indicated rapidly decrease of hatchabilities from 19 to 21 day of incubation in BE1, the side-up, the sharp-end windowed eggs. In contrast, the hatchability of blunt-end windowed eggs(BE(2)) from which the inner shell membrane was removed rapidly decreased at 12 day of incubation. Perhaps the removal of inner shell membrane was negatively influenced for the embryo survival.

DISCUSSION

Because moisture is lost from the egg through the shell during the incubation process, the egg contents reduce and the size of air cell increase. After 19 days of incubation the air cell usually occupies one-third of the egg. It is deeper on one side than the other. The inner cell membrane was removed from the blunt end windowed eggs experiment. Therefore, when the air cell was eliminated from the egg, the

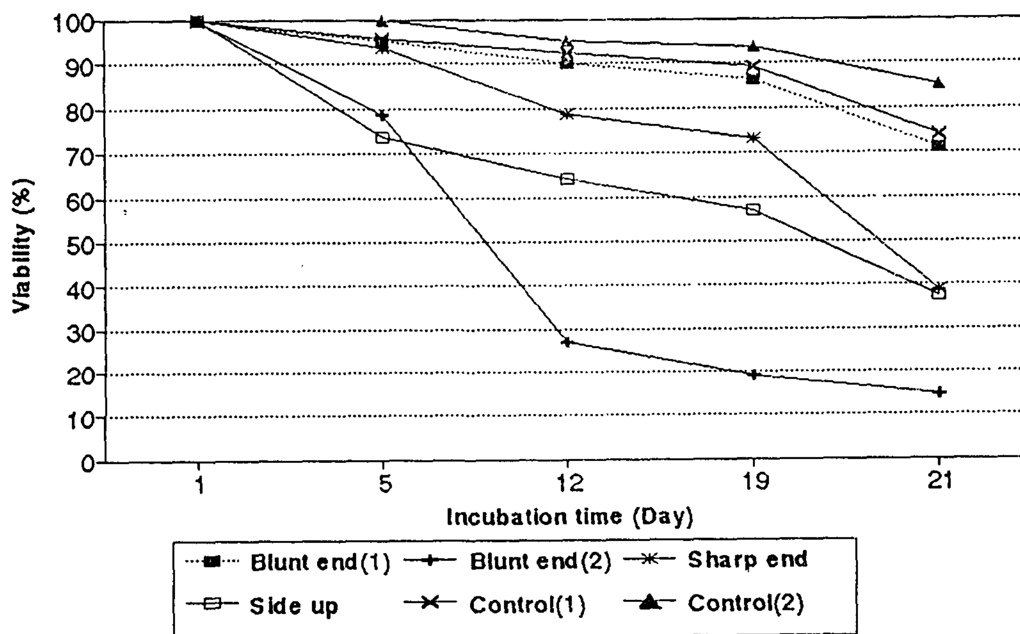

Figure 3. Diagram of viability comparision for the egg window positions.

embryos were slightly dry and the development of embryos stopped. The survival rate of embryos in BE2 rapidly decreased after 12 day incubation. Although the hatchability of BE1 was 71.3%, it's not significantly different from control group (Table 1).

These extraembryonic membrane consist of four kinds of membrane: amnion, chorion, yolk sac membrane, allantois. If one of the membranes is injured during the development of embryo, the developing of embryo will hampered. Because a number of Primordial Germ Cells (PGCs) are circulated in the inner embryonic blood vessel at day 2.5 incubated embryo and the circulating PGCs begin to penetrate into embryonic gonad ridge at day 3.0 embryo (Hong et al., 1995), the egg shell has to be opened and sealedat day 2.5 of incubation if PGCs are used as a vector for transgenic chicken.

The hatchabilities of sharp-end or/and side-up windowed eggs (Table 2, 3) is lower than the hatchability of BE(1) experiment (Table 1). Perhaps the reasons for decreased hatchabilities are trauma and injury of embryos or extraembryonic membrane during the following experiment steps: 1) puncture of egg shell, 2) sealing of window, 3) lowered egg temperature during the treatment. The proportion (%) of dead embryo in control (1) was higher than that in control (2). Maybe the reasons for this lower hatchability in the control experiment are as follows: 1) adhesion of embryo to eggshell during the unrotating procedure, 2) decrease of egg temperature during the treatment in the clean bench.

Therefore, when the hatchability of side-up windowed eggs was compared with the sh-

arp-end windowed eggs, the hatchabilities were not significantly different. In the process of opening the egg shell and injection into the blood vessel, the treatment of side-up windowed eggs is more difficult than the other methods. Injection of DMEM through BE1 was impossible due to obscurity of inner cell membrane. As a result, 2 μL of DMEM was injected into embryonic blood vessel through the sharp-end window. The hatchability of DMEM injected eggs is about 17.0%. Both survival rate and hatchability of DMEM microinjection eggs were lower than the only sharp-end windowed eggs. One of the factors affecting the mortality of the embryos may be the hemorrhage from microinjected blood vessels in spite of injected air bubbles.

The results of this experiment implicated that hatchability would be improved by some factors as follow: 1) egg transfer condition from a rotated incubator to the other unrotated incubator, 2) shortening the waiting time at room temperature, 3) suppressive methods of hemorrhage, 4) minimization of embryo trauma by micro-needle, 5) sealing material which prevents the egg drying, 6) incubation conditions including humidity percent and rotating angle. Therefore, this sharp-end windowed egg system will be useful for the production of germline chimera chicken through primordial germ cell-transfer.

적 요

이 연구의 목적은 주입구의 위치에 따른 병아리 배아의 생존율을 서로 비교하고 가장 적합한 주입구 위치를 찾기 위하여 실시되었다. 멸균처리된 핀셋을 사용하여 난각의 첨단부와 둔단부 그리고 옆부분에 주입구를 각각 만들었다. 연구 결과, 둔단부에 주입구(BE1)를 만든 수정란의 발생율이 가장 높았으나 내부난각막이 불투명하여 혈관내 미세주입이 어렵다. 따라서 본 연구에서는 첨단부에 주입구를 만든 다음 이 주입구를 통하여 약 2 μL의 DMEM 용액을 2.5일령된 배자의 혈관에 주입하였고, 주입부위의 출혈을 막기 위해 DMEM 용액을 주입한 후 공기방울을 넣은 결과 생존율이 약 17.0% 이었다. 따라서 이러한 주입구에 의한 방법은 생식세포가 조작된 germline chimera 또는 형질전환닭을 생산하는데 매우 유용한 시스템으로 이용될 수 있을 것이다.

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