Improvement of Hatchability of Chicken Eggs Injected by Blastoderm Cells[1]

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ABSTRACT In our first experiment, we studied the effect of injection method of blastoderm cells (BC) into the subgerminal cavity of White Leghorn embryos on hatchability of chicken chimeras. Freshly laid eggs were injected through a hole made in the equatorial plane of the eggshell (Method A). In Method B, eggs were stored pointed end down for 5 to 7 d prior to injection, and a hole was cut in the blunt end of the eggshell. An advantage of Method B was that the early embryonic mortality was reduced ($P \leq 0.01$) and resulted in higher hatchability (41.0%; 43/105) than Method A (9.8%; 14/143). In the second experiment, we studied chicken hatchabililty as influenced by windowing (no hole, Group 1; hole in the equatorial plane, Group 2; hole in the blunt end of egg, Groups 3 and 4) and egg turning (Groups 1 and 4) or not (Groups 2 and 3) during incubation. The hatchability percentages were as follows: 67.9 (Group 1) 0.0, (Group 2) 23.3, (Group 3), and 56.8 (Group 4). A statistically significant difference ($P \leq 0.05$) was noted between Group 1 or 4 and the other groups. We found no statistically significant differences in the weight changes (g) but did note certain differences in the egg weight loss (%) among different egg treatments. In the third experiment, we investigated the influence of origins of BC donors: Rhode Island Red (RIR), Barred Plymouth Rock (BPR), and Green-legged Partridgelike (GP) on hatchability of putative and somatic chimera chickens. The hatchability of chimeras was dependent on the adequate assortment of BC of the donor and ranged from 7.4% (RIR) to 56.1% (GP). In the case of BC injection of the GP breed, good hatchability was accompanied by very high percentage (86.9; 20/23) of somatic chimeras.

(*Key words*: blastoderm cells, chick, chimera, hatchability)

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INTRODUCTION

In the past, considerable attention has been given to the production of transgenic poultry. Many attempts have been made to produce transgenic birds for genetic improvement, production of more useful products, and conservation of foundation stocks of poultry. To introduce exogenous genes into poultry, certain methods have been reported. They include: 1) viral vector (Petropoulos et al., 1992; Thoraval et al., 1995), 2) direct DNA microinjection into the germinal disk (Love et al., 1994; Naito et al., 1994; Sherman et al., 1998), 3) in vivo embryo lipofection or electroporation (Muramatsu et al., 1997), and 4) use of primordial germ cells (Naito et al., 1998; Ono et al., 1998; Ono and Machida, 1999) or blastodermal cells (BC) (Petitte et al., 1990; Brazolot et al., 1991; Kino et al., 1997). In our laboratory, the transfer of donor BC into windowed eggs has produced chicken chimeras. Manipulation of BC is very useful for producing transgenic chickens because Stage X blastoderms are transferred into recipient embryos and form somatic and germline chimeras. They can be maintained in culture for several days and genetically modified in vitro (Etches et al., 1997). The newly laid and unincubated egg is accessible to the population of BC, and this process can be performed without killing the mother.

These properties make BC ideal vectors for introducing genetic modification into the germline. For this reason, the method of producing transgenic birds by BC injection has been used frequently. However, the percentage of identified chicken chimeras (e.g., by plumage color) is low. One of the causes is the low survival rate of the treated embryos (Petitte et al., 1990; Thoraval et al., 1994; Pokorny, 1999). In many reports, no data on survival rate have appeared, and only the percentage of somatic or genetic chimeras in relation to the total potential chimeras is given. Some published experimental results show substantial mortality of recipient embryos, particularly in the first days after injection (Thoraval et al., 1994).

In the present study we investigated the influence of method of injection (A or B), and origin of BC donors

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Abbreviation Key: BC = blastoderm cells; RIR = Rhode Island Red; BPR = Barred Plymouth Rock; GP = Green-legged Partridgelike; WL = White Leghorn; CMF-PBS = calcium-magnesium free PBS.

[Rhode Island Red (RIR), Barred Plymouth Rock (BPR), and Green-legged Partridgelike (GP)] on hatchability of putative and somatic chimera chickens.

MATERIALS AND METHODS

Experimental Birds

White Leghorn (WL) chickens maintained in our laboratory were used as recipients because they were homozygous at the dominant white locus (*II*). Donor BC were obtained from eggs of chickens that were homozygous recessive at the white locus (*ii*). We used BPR and RIR chickens from a local commercial poultry breeder and GP chickens. The GP chickens are a native Polish breed of layers and have been kept in a closed population over 50 generations. They have light body weight (1.5 to 1.8 kg at 20 wk of age), brown-grey plumage, and bright green shanks. These chickens and their hybrids demonstrate high resistance to disease and ability to produce in different environmental conditions.

General Methods

Stage X (Eyal-Giladi and Kochav, 1976) blastoderms from freshly laid donor eggs were prepared with filter paper rings (Petitte et al., 1990). Blastoderms from 20 eggs were isolated from yolk and washed several times with calcium- and magnesium-free PBS[3] (CMF-PBS) to remove as much yolk as possible. They were then dispersed for 7 min at 20 C in CMF-PBS supplemented with 0.05% (wt/vol) trypsin[3] and 0.04% (wt/vol) EDTA[3] and by repeated aspirations of the medium into a Pasteur pipette. After dispersion, the cells were centrifuged for 5 min at a relative centrifugal force (RCF) of $300 \times g$ and then washed three times with CMF-PBS supplemented with 10% (vol/vol) fetal bovine serum.[4] Each supernatant was removed, and the cells from several embryos were resuspended in Dulbecco's Modified Eagle Medium[4] prior to injection.

On the day of injection, the shell was swabbed with 70% (vol/vol) ethanol, a window of 1.0 to 1.2 cm in diameter was made, and then a small piece (approximately 0.4 × 0.4 cm) of the membrane (directly over the blastoderm) was carefully removed to expose the embryo. Approximately 1,200 to 2,000 donor BC in 1 to 2 μL medium was injected into the subgerminal cavity of recipient embryo. Micropipettes of 80 to 100 μm, used for cells injection into the recipients, were made from borosilicate glass capillaries (GC100T-10)[5] drawn out with a micropipette puller (PUL-1)[6] and were sterilized by UV radiation. The micropipette was fitted to a micromanipulator (Prior type).[7] All of the procedures for injection and embryo manipulation were carried out under sterile conditions.

Each window was sealed with an adhesive tape. The eggs were incubated in a conventional forced-air apparatus maintained at 37.7 C and 50% relative humidity for 19 d and thereafter were transferred to a hatcher at 37.0 C and 85% relative humidity until hatched.

Data on hatchability, embryonic mortality, and egg weight losses (percentage) were analyzed by a chi-square contingency test (Laughlin and Lundy, 1976). Significant differences between egg weights were statistically verified by analysis of variance. The means were separated by Duncan's new multiple-range test (Duncan, 1955).

Experimental Procedure

In the first experiment, we studied the effect of injection method (A or B) on hatchability of chicken chimeras. Freshly laid eggs were injected into the subgerminal cavity, through a hole made in the equatorial plane of the eggshell (Method A). In Method B certain modifications were introduced: 1) eggs were stored pointed end down for 5 to 7 d prior to injection and 2) a hole was cut in the blunt end of the eggshell (above the air chamber). The study comprised 143 and 105 embryos treated with Method A or B, respectively.

In the second experiment, we studied the influence of windowing on chicken hatchability. The groups (43 to 56 eggs/group) were as follows: 1) control, not treated eggs, turned during incubation; 2) a hole was made in the equatorial plane of eggshell, and eggs were not turned; 3) a hole was made in the blunt end of egg, and eggs were not turned; 4) a hole was made in the blunt end of egg, and eggs were turned during incubation.

Eggs were incubated in a BIOS-MIDI[8] experimental incubator. The eggs were candled on Days 10 and 18, and infertile eggs and dead embryos were eliminated. We calculated the percentage of dead embryos from Days 0 to 10, 11 to 18, and 19 to 21; never-hatched embryos and hatchability from treated eggs were also calculated. Individual egg weights before setting and on Days 5, 10, 15, and 18 of incubation were determined; weight changes of eggs during incubation (percentage weight loss) were also measured.

In the third experiment, the influence of origins of BC donor on hatching rate of the putative and somatic chicken chimeras was studied. Donor BC were obtained from eggs of BPR, RIR, and GP chickens and were injected into recipient WL embryos, according to the procedure described in Method B. The hatched chicks were visually observed to determine the incorporation of donor cells into somatic tissues by estimating the extent of black pigmentation in the down of the chimeras.

RESULTS

Table 1 presents the effect of two injection methods (A and B) of BC on chicken hatchability. Hatchability

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TABLE 1. Influence of injection method of blastoderm cells on chicken hatchability

	Repetition						
Method[1]	1	2	3	4	5	6	Total
A							
Number of embryos treated	53	22	25	43			143
Hatchability (%)	7.5	4.5	16.0	11.6			9.8[B]
B							
Number of embryos treated	15	17	18	16	16	23	105
Hatchability (%)	46.7	35.3	50.0	25.0	37.5	47.8	41.0[A]

[A,B]Means with no common superscript differ significantly between groups ($P \leq 0.01$).

[1]Method A = freshly laid eggs were injected into the subgerminal cavity, through a hole made in the equatorial plane of the eggshell. Method B = eggs were stored pointed end down for 5 to 7 d prior to injection, and a hole was cut in the blunt end of the eggshell (above the air chamber).

after injection according to the Method A was 9.8% of the treated embryos and ranged from 4.5 (replication 2) to 16.0% (replication 3). In Method B the mean hatchability from six repetitions was 41.0% (from 25.0 to 50.0%). The difference between Methods A and B was significant ($P \leq 0.01$).

Figure 1 demonstrates the effect of injection method of BC on embryonic mortality during incubation. Three peaks of embryo mortality in the third, ninth, and eighteenth days of incubation were noted, regardless of the injection method used. Higher embryo mortality was found on the third day of incubation. However, in that period, 51.0% of embryos died under injection Method A, whereas only 20.0% of embryos died when injection Method B was used. On the ninth and eighteenth days of incubation, a lower mortality was observed, i.e., 14 and 8% or 6 and 14% for Methods A or B, respectively.

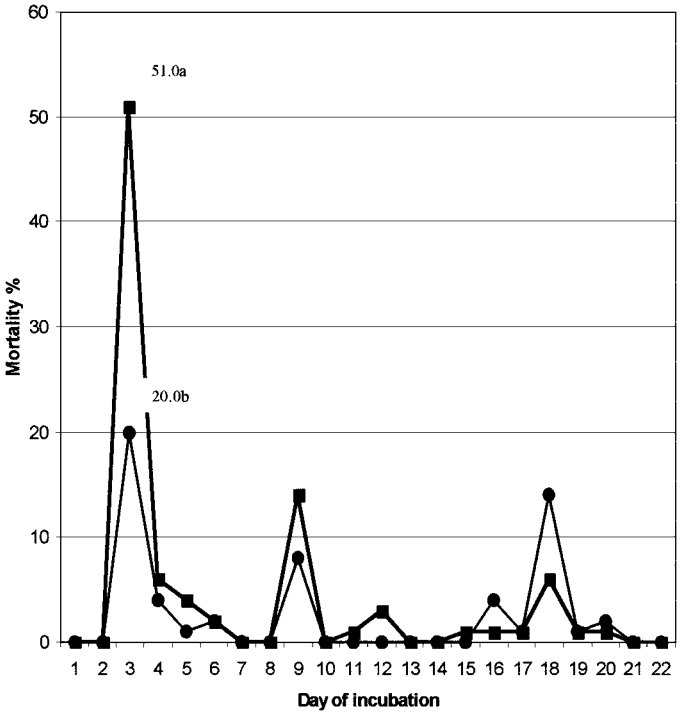

FIGURE 1. Effect of injection method of BC on embryonic mortality. Method A (■), freshly laid eggs were injected into the subgerminal cavity, through a hole made in the equatorial plane of the eggshell; Method B (●), modified donor eggs were stored pointed end down for 5 to 7 d prior to injection (a hole was cut in the blunt end of the eggshell). Embryonic mortality was calculated from 143 and 105 embryos treated with Method A or B, respectively. [a,b]Significantly different ($P \leq 0.01$) percentages of mortalityat the third day of incubation for embryos treated with Method A or B.

In the second experiment (Table 2), the highest hatchability (67.9%) was found in Group 1 (control = no hole in the eggshell, and eggs were turned). In the groups for which a hole was made in the blunt end of the egg, the hatchability was 56.8 and 23.3% for Groups 4 (turned eggs) and 3 (no turned eggs), respectively. In Group 2 for which the hole was made in the equatorial plane of eggshell (no turned eggs) the hatchability was 0.0% (no live chicks). The early, middle, and late mortalities in this experiment ranged from 17.8 (Group 1) to 44.0% (Group 2), from 1.8 (Group 1) to 5.3% (Group 2), and from 4.5 (Group 4) to 40.7% (Group 2), respectively. We noted no statistically significant differences in the weight changes (g) among eggs treated differently (Table 3). Certain differences were found in the percentage egg weight loss during incubation. The greatest was in Group 1, i.e., 3.0, 4.8, and 6.3% until the fifth, tenth, and fifteenth days of incubation, respectively. On the eighteenth day of incubation, however, no significant

TABLE 2. Mortality and hatchability of chicken embryos from differently trated eggs

	Group[1]			
	1	2	3	4
Number of eggs set	56	59	43	44
Dead embryos (%)				
0 to 10 d	17.8	44.0	27.9	25.0
11 to 18 d	1.8	15.3	14.0	9.1
19 to 22 d	12.5	40.7	27.9	4.5
Hatchability (%)	67.9[a]	0.0[b]	23.3[b]	56.8[a]

[a–c]Means with no common superscript differ significantly between groups ($P \leq 0.05$).

[1]Group 1= control, untreated eggs, turned during incubation; Group 2 = a hole was made in the equatorial plane of eggshell, and eggs were not turned; Group 3 = a hole was made in the blunt end of egg, and eggs were not turned; Group 4 = a hole was made in the blunt end of egg, and eggs were turned during incubation.

TABLE 3. Weight changes during successive days of incubation of differently treated eggs

	Group[1]			
Day	1	2	3	4
	(g)			
0	62.7 ± 5.1[a]	63.0 ± 5.8[a]	63.9 ± 5.2[a]	64.2 ± 5.8[a]
5	60.2 ± 4.1[a]	60.6 ± 6.1[a]	62.4 ± 5.2[a]	61.7 ± 4.9[a]
10	59.7 ± 5.1[a]	60.2 ± 5.4[a]	61.6 ± 4.7[a]	61.7 ± 5.7[a]
15	58.9 ± 4.7[a]	59.3 ± 5.4[a]	59.9 ± 4.7[a]	60.6 ± 5.3[a]
18	57.2 ± 4.8[a]	58.5 ± 3.7[a]	58.4 ± 4.1[a]	58.2 ± 4.9[a]
	(%)			
0 to 5	3.0 ± 0.6[a]	2.8 ± 0.4[b]	2.8 ± 0.3[b]	2.8 ± 0.2[b]
0 to 10	4.8 ± 1.9[a]	4.4 ± 1.4[ab]	3.9 ± 0.7[b]	3.9 ± 0.9[b]
0 to 15	6.3 ± 1.4[ab]	6.5 ± 1.1[a]	5.9 ± 0.8[b]	5.9 ± 0.9[b]
0 to 18	9.2 ± 1.3[a]	9.3 ± 3.8[a]	8.8 ± 1.2[a]	9.1 ± 2.5[a]

[a,b]Means ± SD with no common superscript differ significantly between groups ($P \leq 0.05$).

[1]Group 1 = control, untreated eggs, turned during incubation; Group 2 = a hole was made in the equatorial plane of eggshell, and eggs were not turned; Group 3= a hole was made in the blunt end of egg, and eggs were not turned; Group 4 = a hole was made in the blunt end of egg, and eggs were turned during incubation.

differences in percentage egg weight loss were noted among groups (from 8.8 to 9.3%).

Table 4 presents the influence of origins of BC donor on hatching rate of the chick chimeras. The hatchability ranged from 7.4 (RIR) to 56.1% (GP). For BC injection of the GP breed, good hatchability was accompanied by very high percentage of somatic chimeras. Among 23 chicks, 20 (86.9%) had black spots in their plumage, which was a phenotypic marker of chimerism.

DISCUSSION

There are several factors that increase the frequency of somatic and germline chimeras: exposure of recipient embryos to gamma irradiation (Carsience et al., 1993; Kagami et al., 1995), injection of recipient embryos with the cytotoxic drug busulphan (Vick et al., 1993), change in the volume of the cell suspension injected (Naito et al., 1991), depth of BC injection (Maeda et al., 1997), and adequate assortment of recipients and donors of BC (Carsience et al., 1993). Most important, however, is the adequately high survivability of the treated recipient embryos after injection of donor BC.

The published reports on this topic indicate that hatchability of the injected embryos is very low (Petitte et al., 1990; Maeda et al., 1997; Pokorny, 1999). For example, only 2.8% of 408 WL eggs hatched after windowing compared with 61 to 92% for unwindowed eggs (Speksnijder et al., 1998). Thorval et al. (1994) observed a high degree of mortality in injected embryos (5.0 to 7.3% embryos survived to hatching). Better results (hatchability 33.0%) were reported by Speksnijder et al., (1998) as a result of simple modifications to the windowing technique; however, they did not describe them.

Viability of the embryos injected with foreign cells rapidly decreased during the first days of incubation (Ono et al., 1994; Pokorny, 1999). A similar phenomenon was noted for Method A in the current study (Figure 1), but an advantage of Method B was that the early embryonic mortality was significantly reduced and resulted in a higher hatchability (41.0%; 43/105) than with the conventional method (9.8%; 14/143). The mean hatchability (41.0%) calculated from six replications (Table 1) was close to the best results reported by Naito et al. (1991) and Kino et al. (1997), i.e., 8.6 to 40.3% and 25.6 to 40.7%, respectively. However these authors used another method of incubation and transferred 4-d-old embryos to a large recipient surrogate eggshell. Method B proposed in this report is considerably easier than the transfer of embryos to surrogate eggshells, as was used by Naito et al. (1991) and Kino et al. (1997).

The exact reason for the decline in hatchability of the injected embryos is unknown. Several probable causes can be identified: the windowing procedure, injection per se, and potentially inadequate water vapor transmission rate through the eggshell partially sealed with tape. Analysis of data presented in Figure 1 indicated that the second peak of mortality in the ninth day of incubation was characteristic of injected embryos, and mortality from 8 to 14 d of incubation is normally very low (Romanoff, 1972). According to Petitte et al. (1990), hatchability of eggs containing early stage embryos is substantially reduced by the windowing procedure. On the other hand, Han et al. (1994) found no effect of windowing on embryonic viability. Our findings presented in Table 2 indicate that windowing per se does not significantly affect early embryonic mortality. Windowing at the equatorial plane of the eggshell and static incubation (Group 2) significantly increased ($P \leq 0.05$) early and late embryonic mortality. Opening of the eggshell at the blunt end (Group 4), however, had no significant effect on hatchability when compared with Group 1 (not treated). When the windowed eggs (Group 3) were not turned, the hatchability was reduced by more than 30%, in comparison with the windowed and turned eggs (Group 4).

TABLE 4. Influence of origins of donor of blastoderm cells on hatching rate of the chicken chimeras

Donor	Recipient	Number of embryos treated	Hatched chicks (%)	Number (%) of chimeras	
				Putative	Somatic
Barred Plymouth Rock	White Leghorn	71	33.8[a]	22 (91.7)	2 (8.3)
Rhode Island Red	White Leghorn	68	7.4[c]	5 (100)	. . .
Green-legged Partridgelike	White Leghorn	41	56.1[b]	3 (13.0)	20 (86.9)

[a–c]Means with no common superscript differ significantly between groups ($P \leq 0.05$).

The differences observed in mortality and hatchability of eggs treated differently could not be explained by egg weight change during incubation (Table 3). Different early embryonic mortalities in Groups 2 (44.0%), 3 (27.9%), and 4 (25.0) were accompanied by the same egg weight loss (2.8%). Significant differences in the percentage weight loss between Group 1 (67.9% hatchability), and Group 2 (0.0% hatchability) were not found, except during the initial period until the fifth day of incubation. It seems, however, that the differences in hatchability and mortality of embryos treated by Method A or B (Table 1 and Figure 1) can be associated with the protective function of air chamber. Windowing of the eggshell above the air chamber, which naturally isolates the embryo (and increases during incubation), is less detrimental than opening the eggshell at its equatorial plane.

The results of our study (Table 4) confirm the findings of other authors (Carsience et al., 1993; Petitte et al., 1993; Ono et al., 1995) that the hatchability of chimeras is dependent on an adequate assortment of BC from the donor and recipient. However, it is difficult to explain why the injection of BC into GP eggs was so successful, whereas the use of RIR BC drastically reduced the percentage of hatchability and the number of somatic chimeras among WL recipient embryos. Presumably the integration of the donor cells with those of the recipient, being an indispensable condition of chimera development, is dependent on mutual synchronization of their embryogenesis rates. Genetic differences exist in the early embryogenesis rate of the chicken of various origins. For example, heavier birds tend to present fewer somites during the early phase of embryogenesis (Coutinho et al., 1993; Schmidt et al., 1999). In the present situation, it can only be stated that RIR chickens have the highest body weight among the three breeds we used as BC donors and are substantially heavier than WL chickens.

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