Culture of Chicken Embryos in Surrogate Eggshells

S. Borwornpinyo, J. Brake, P. E. Mozdziak,[1] and J. N. Petitte

Department of Poultry Science, North Carolina State University, Raleigh, North Carolina 27695

ABSTRACT The chick embryo is a classical model to study embryonic development. However, most researchers have not studied the effect of embryonic manipulation on chick hatchability. The objective of this study was to determine the effect of egg orientation and type of sealing film on the hatchability of cultured embryos. Windows were made in the small end of recipient surrogate chicken eggshells, and donor embryos were placed into the recipient eggshell for the first 3 d of incubation. Survival over the first 3 d was maximized ($P < 0.05$) when windowed eggs sealed with Saran Wrap were positioned with the window-end down compared with window-end up. Three-day-old cultured embryos were transferred into recipient turkey eggshells, sealed with cling film, and cultured until hatch. Water weight loss of the surrogate eggshell cultures regardless of cling film type was not significantly different from control intact eggs. The embryos cultured in turkey eggshells and sealed with Handi Wrap exhibited higher hatchability (75% ± 10.2%) than cultures sealed with Saran Wrap (45.2% ± 13.8%). Hatchability of control intact eggs (86.4% ± 5.3%) was not significantly ($P > 0.05$) different from the hatchability of eggs sealed with Handi Wrap, which suggested that Handi Wrap was an excellent sealant for chick embryos cultured after 3 d of incubation.

(*Key words*: surrogate eggshell, avian embryo culture, transgenic bird, hatchability)

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INTRODUCTION

Since the early 1980s many attempts have been made to produce transgenic chickens as bioreactors for pharmaceutical protein production in eggs (Harvey et al. 2002a; Ivarie, 2003). Early progress in creating transgenic birds was hampered by nonexistent procedures to successfully culture avian embryos until hatch. Development of the avian embryo depends upon the maternal nutrition packaged into the eggshell. Nonetheless, culture of preincubated embryos in surrogate eggshells as culture vessels successfully produces live birds that reached sexual maturity (Ono and Wakasuki, 1984; Rowlett and Simkiss, 1985, 1987). It was not until 1988 that Perry developed a complete culture system supporting the chick embryos from the single-cell stage to hatching (Perry, 1988). Perry's (1988) successful culture required 3 different sequential systems. In system I, the fertilized ovum was cultured in vitro for 24 h to allow formation of the blastoderm. In system II, the cultured blastoderm was transferred into small recipient chicken eggshells and cultured for 3 d to complete embryogenesis, and for the remainder of incubation (system III), the embryo was transferred into large recipient chicken eggshells containing an airspace. However, the hatching rate of fertilized ova in Perry's complete culture system was reported to be only 7% (Perry, 1988). Subsequently, culture procedures have been developed to improve hatchability to 34% (Naito and Perry, 1989; Naito et al., 1990) and adapted for other avian species, such as quail (Ono et al., 1994; Naito et al., 1995; Kamihira et al., 1998). In 1994, DNA microinjection of fertilized ova was first applied in the chicken, and the manipulated ova were cultured using Perry's culture system (Love et al., 1994), but a more effective method for genetic modification of avian species has been the transduction of embryos with retroviral vectors (Petitte and Mozdziak, 2002; Kamihira et al., 2004; McGrew et al., 2004; Sang, 2004). Viral injection into embryos from freshly laid eggs was performed through a small window in the eggshell, which was sealed (Salter et al., 1986; Bosselman et al., 1989; Speksnijder and Ivarie, 2000; Harvey et al., 2002a,b; Andatch et al., 2004). The windowing technique is simple but often results in low hatch rates (Speksnijder and Ivarie, 2000). Recently, an improved sealing technique to avoid trapped artificial air bubbles during sealing, resulted in 45% hatchability (Andatch et al., 2004). In contrast to simple windowed eggs, surrogate eggshells provide a potentially better accessibility for the manipulation of the embryos and allow for multiple operations to be performed during incubation (Mozdziak et al., 2003).

In using surrogate eggshell methods for unincubated freshly laid eggs involving 2 sequential recipient eggshells, previous researchers (Rowlett and Simkiss, 1987; Perry, 1988) have not studied the influence of culture

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[1]To whom correspondence should be addressed: pemozdzi@unity.ncsu.edu.

orientation in system II or the type of covering materials for the cultures in systems II and III. Rotation of the embryos and sealing of the cultures may have a profound impact on hatchability. Thus, the first objective of this study was to determine the effect of window orientation of surrogate eggshells and type of sealing film on the survival of embryos from freshly laid eggs in surrogate eggshell system II. The second objective of the study was to evaluate the effect of the cling film type, as covering material, on the survival and hatchability of embryos in surrogate eggshell system III.

MATERIALS AND METHODS

Fertile Egg Source

Freshly laid fertile eggs were obtained from broiler breeder flocks,[2] 30 to 57 wk of age, and were maintained at the Chicken Educational Unit, North Carolina State University. All procedures involving animals were approved by the North Carolina State University, Institutional Animal Care and Use Committee. The breeder flocks (60 females and 6 males) were housed in slat-litter pens or in individual cages. The hatching eggs were collected and placed in egg flats. Dirty or cracked eggs were discarded.

Recipient Chicken and Turkey Eggshells

All recipient chicken (*Gallus domesticus*) eggshells were prepared from newly laid eggs that were 3 to 4 g heavier than donor eggs. All recipient chicken eggs were wiped with 70% alcohol. A 32-mm diameter opening was made at the pointed end of the egg using a small hand-held electric drill.[3] The yolk and albumen of recipient eggs were discarded. The empty eggshells were first washed on the outside and rinsed on the inside with distilled water to remove adhering albumen. The eggshells were placed with the open end down in a glass baking dish lined with a moistened paper towel to humidify the shell membrane.

Turkey, *Meleagris gallopavo*, eggs (Nicholas Strain) that were 35 to 40 g heavier than the donor chicken eggs were selected for surrogate eggshells. The preparation of recipient turkey eggshells was the same as recipient chicken eggshells except that a 42- to 45-mm diameter opening was made at the blunt end of the eggshell.

Surrogate Eggshell Culture in System II

The culture methods were modified from Perry's (1988) surrogate eggshell culture system II. Donor chicken eggs weighing between 55 to 60 g were wiped with 70% alcohol. To minimize bacterial and fungal contamination of cultures, transfer procedures were performed in a laminar airflow hood. The donor eggs were cracked into a plastic drinking cup modified to a 20-mL holding volume (Figure 1A).

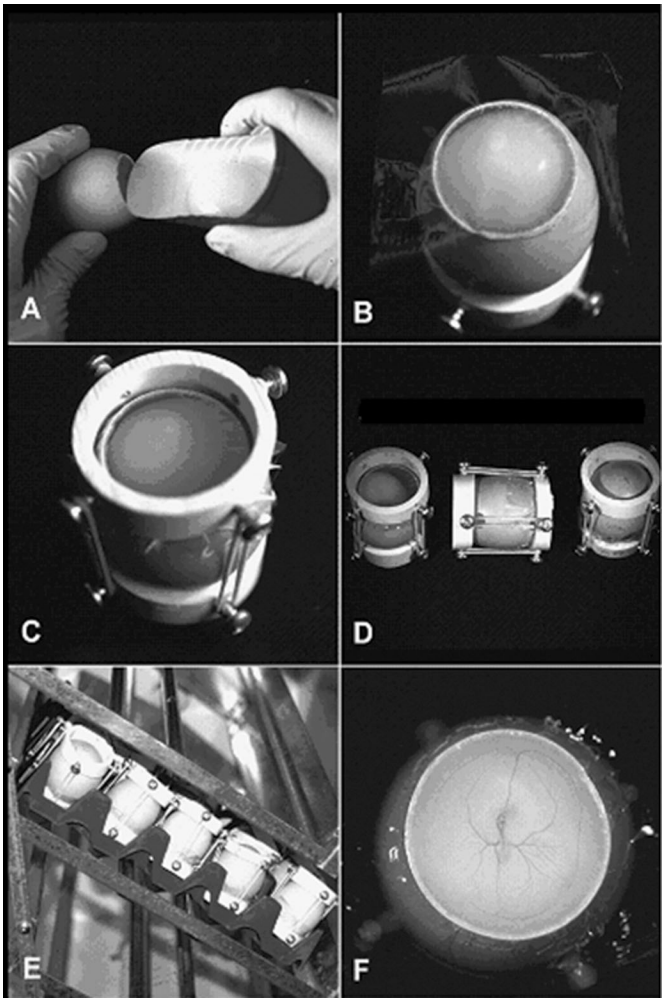

FIGURE 1. Culture procedures of embryos from freshly laid eggs in surrogate chicken eggshells during the first 3 d of incubation. A. Transfer of donor embryo into recipient chicken eggshell. B. Covering of recipient eggshell with cling film. C. Sealed recipient eggshell with donor embryo. D. Three different eggshell orientations examined during the first 3 d of incubation. E. Incubation position of recipient chick eggshells containing donor embryos (rotated 45° once per hour). F. Chick embryo in recipient eggshell after 3 d of incubation.

Only embryos from freshly laid eggs were selected for culture. The entire contents of the fertile egg were poured into a suitable recipient chicken eggshell (Figure 1A). Albumen (thick and thin) from other freshly laid eggs was added to fill the shell. The rationale for filling the shells was to eliminate air bubbles in the culture after sealing the window with a 5 × 5 cm piece of Saran Wrap or Handi Wrap (Figure 1B). Table 1 reports the effective permeability of Saran Wrap and Handi Wrap.[4] After being sealed with albumen to adhere the wrap to the shell, the window was secured using a pair of PVC plastic rings embedded with 4 screws with rubber bands extending from the rings (Figure 1C). The reconstituted eggs were oriented in 3 different positions (Figure 1D). The up posi-

[2]Arbor Acres, feather-sexable strain, Glastonbury, CT.
[3]Dremel, Racine, WI.
[4]Dow Chemical, Midland, MI.

TABLE 1. Effective permeability of cling film types used as covering materials in surrogate eggshell culture systems II and III[1]

	Thickness (μm)	Effective permeability[2]		
		O_2	CO_2	H_2O[3]
Saran Wrap	500	3.0	19.96	0.90
Handi Wrap	500	1600	7920	4.0

[1]Data from Dunn et al. (1981).

[2]Effective permeability = permeability (in mL/100 in.^{2} per 24 h/atm).

[3]Effective permeability to water vapor = permeability (in g/100 in.^{2} per 24 h/atm).

tion was eggs placed with the sealed opening uppermost. The side position was with eggs parallel to the horizontal axis in the incubator rack. The down position was with the sealed window placed bottommost. Surrogate eggshell cultures were incubated in the incubator at 37.5°C and 60% RH; eggs were rocked through an angle of 90° at hourly intervals for 3 d (Figure 1E). Embryonic mortality was monitored daily. Embryos were considered to be dead when the outer layer of the blastoderm stopped development, the heart did not beat, and the embryonic circulation collapsed.

Surrogate Eggshell Culture in System III

The technique for the transfer of 3-d-old embryos to larger surrogate eggshells is reported to be particularly difficult (Rowlett and Simkiss, 1987; Perry, 1988). Therefore, transfer of an embryo from the surrogate chicken eggshell to the surrogate turkey eggshell was recognized to be a critical step in the process. First, the eggs in system II were positioned with the window uppermost to allow the embryos to float close to the window. A portion of the cling film was opened, and approximately 5 mL of albumen was poured from the surrogate chicken eggshell into the surrogate turkey eggshell (Figure 2A). For the transfer, the entire cling film was removed from the chicken eggshell, and any air bubbles were eliminated from the system III cultures. Next, the edge of the reconstituted chicken egg was trimmed with scissors to make the opening smooth (Figure 2B). The turkey eggshell containing the 5 mL of albumen was rolled to wet the inner shell membrane. To perform the transfer, the cut edge of the chicken and the turkey eggshells were placed side-by-side and tilted toward each other until the albumen from both sources met (Figure 2C). The embryos and their contents were rapidly (approximately 1 s) and gently poured into the surrogate turkey eggshell (Figure 2D). Using this procedure, all embryos were transferred successfully without damage and most transferred embryos were on top and centered inside the surrogate turkey eggshell (Figure 2E). Embryo position was important because any embryos remaining on the bottom of the turkey shell did not float and subsequently died.

Finally, the turkey shells were sealed with a layer of a 6.5 × 6.5 cm cling film that was glued with albumen applied around the outside area at the cutting edge (Figure 2F) and secured with a pair of plastic rings (50 mm

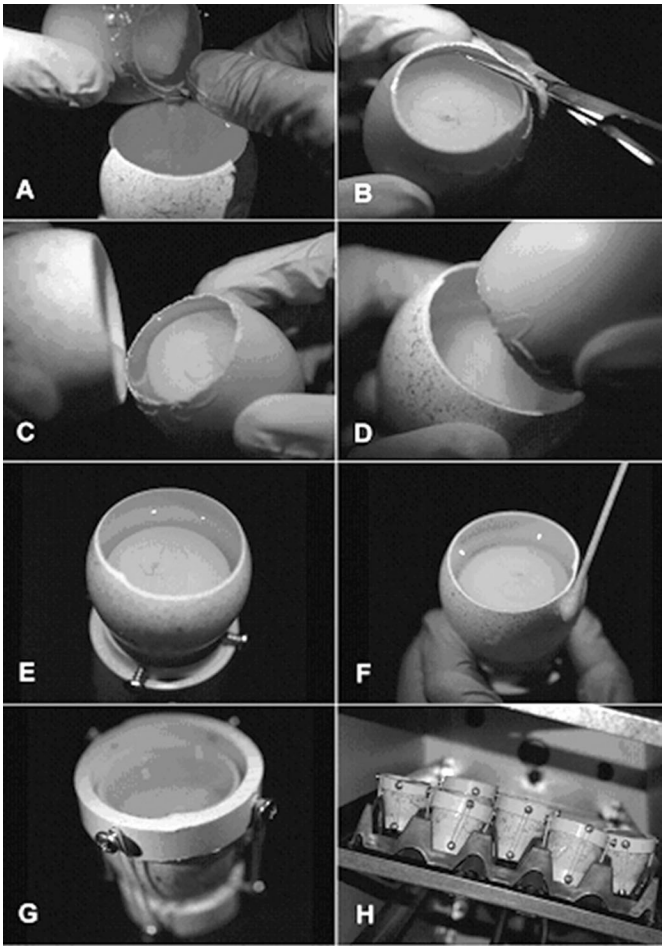

FIGURE 2. Culture procedures of 3-d-old embryos in surrogate turkey eggshells through hatching. A. Transfer of donor chick albumen from recipient chick eggshell to recipient turkey eggshell. B. Trimming of recipient chicken eggshell before transfer of donor embryo. C. Beginning of transfer of donor chick embryo to recipient turkey eggshell. D. Conclusion of transfer of donor chick embryo to recipient turkey eggshell. E. Donor chick embryo in recipient turkey eggshell. F. Coating rim of recipient turkey eggshell with albumen. G. Fully prepared recipient turkey eggshell containing donor chick embryo. H. Incubations position of recipient chick eggshells containing donor embryos (rotated 30° once per hour).

in diameter at the cutting site and 35 mm in diameter at the bottom site) and elastic bands (Figure 2G). The surrogate eggs were incubated at 37.5°C and 60% RH and turned through an angle of 30° at hourly intervals. During turning, the albumen inside the surrogate turkey eggshell culture did not touch the cling film. After 18 d of incubation consisting of 3 d in system II and 15 d in system III, eggs were placed in a hatcher at 37.5°C and 60% RH. The cling film was loosened completely around the surrogate turkey eggshell with the first sign of chorioallantoic membrane penetration (internal pipping) around d 19.5 and then covered with a small Petri dish lid (60 × 15 mm). The Petri dish lid made it possible for the chicks to easily emerge from the shells, and it reduced moisture loss immediate posthatch. The chicks were allowed to hatch and crawl out of the shells independently.

Embryonic mortality was monitored once a day through the cling film without removing it. Embryos were

considered to be dead when the area vasculosa did not develop, when there was no heartbeat, when there was no gross movement, and when the circulation collapsed. Intact eggs were used as the experimental control, which were candled at d 7, 14, and 18 of incubation to monitor survival. Dead embryos from the intact eggs were opened to check their developmental stages. All manipulated embryos were visually assessed for fertility during the initial transfer. All infertile embryos at initial manipulation were excluded from analysis. Dead embryos at early stages of development were classified as dead at d 0 of incubation.

Water Loss During Culture and Chick Weight at 1 d of Age

Water loss was monitored daily by weighing the surrogate turkey egg (in system III) at the time of transfer as the reference point for water loss measurements and subsequently daily until d 18 of incubation. Water losses were added as a cumulative water loss for each day of incubation and were expressed as a percentage of the egg weight of the surrogate system III on d 3. Control eggs were incubated under the same conditions as described above with the blunt end up. The control eggs were weighed before incubation as a reference point. Only eggs surviving until d 18 of incubation before transfer to the hatcher were used for statistical analysis. Chicks hatching from the surrogate eggshells or the control eggs were weighed 1 d after hatch.

Statistical Analysis

Numbers of dead and live embryos at d 3 of incubation in system II surrogate eggshell cultures were subjected to χ^2 analysis using Statistical Analysis System (SAS Institute, 1990). Three positions (up, side, and down) or 2 positions (up and down) were analyzed as the main effects on survivability of cultured chicken embryos at d 3 incubation with Saran Wrap and Handi Wrap. Statements of statistical significance were based on $P < 0.05$.

Numbers of dead and live embryos were classified into 4 different incubation periods: in system II, d 0 to 3; in system III, d 4 to 11, d 12 to 18, and 19 to hatching period; and for the overall incubation, d 0 to hatching. The data were subjected to χ^2 analysis using the Statistical Analysis System (SAS Institute, 1990). Three trials and 2 cling-film types, Saran Wrap and Handi Wrap were analyzed as the main effects on survivability and hatchability of cultured chicken embryos. Statements of statistical significance were based on $P < 0.05$.

The daily cumulative percentages of weight loss among 3 treatments of all trials were plotted with the x-axis as incubation time (d) and the y-axis as an cumulative percentage of weight loss. Slopes of each treatment were calculated to analyze the main effects on cumulative percentage of weight loss by using the SAS Procedure PROC GLM (SAS Institute, 1990). Statements of statistical significance were based on $P < 0.05$. The main effect of chick weight was analyzed using the SAS PROC GLM (SAS Institute, 1990). Statements of statistical significance were based on $P < 0.05$.

TABLE 2. The effect of egg orientation on chicken embryo viability at d 3 of incubation in surrogate eggshell system II using Saran Wrap as covering material

Position[1]	n	Viable embryos at d 3 of incubation (%)
Up	45	8.8[b]
Side	45	82.8[a]
Down	45	100.0[a]

[a,b]Data with different superscript differ significantly ($P < 0.05$).

[1]In the up position, eggs were placed with the sealed opening uppermost. The side position was with eggs placed parallel to horizontal axis (parallel to the racks in the incubator). In the down position, eggs were placed with the sealed window bottommost.

RESULTS AND DISCUSSION

The embryonic survival of embryos from freshly laid chicken eggs after 3 d of incubation in surrogate eggshell culture system II is shown in Tables 2 and 3. Embryos cultured in surrogate eggshells sealed with Saran Wrap in the down and side positions survived significantly better than those embryos in the up position ($P < 0.05$). Embryos in down and side positions showed similar survivability and normal development (stages 18 to 19, Hamburger and Hamilton, 1951). Embryos cultured using Handi Wrap as a covering material showed statistically similar survivability as cultures in the up position (85.7%) compared with cultures in the down position (97.5%), but it is likely that embryos are most appropriately cultured in the down position (Table 3).

During the first 3 d in system II, the survival of the eggs sealed with Saran Wrap and Handi Wrap ($P > 0.05$) was not different when cultured in the down position (Table 4). The absolute difference between treatements was 11.8%, but the difference was not statistically significant. The number of observations (n = 42) might have contributed to not revealing any statistically significant differences (Table 3). Between d 3 and 12, embryos cultured with Saran Wrap showed significantly higher mortality than with Handi Wrap ($P < 0.05$; Table 4). The low embryonic death in the Handi Wrap group did not seem to be affected by the initial conditions, which suggests that the method of transferring to a turkey eggshell did

TABLE 3. The effect of egg orientation on viability at d 3 of incubation of chicken embryos from freshly laid eggs in surrogate eggshell system II using Handi Wrap as covering material

Position[1]	n	Viable embros at d 3 of incubation (%)
Up	42	85.7[a]
Down	42	97.5[a]

[a]Values with the same superscript do not differ significantly among positions ($P < 0.05$).

[1]The up position was the eggs placed with sealed opening uppermost. The down position was the sealed window placed bottommost.

TABLE 4. Effect of cling-film type on survival and hatchability of chicken embryos from freshly laid eggs in surrogate eggshell culture system II (d 1 to 3) and system III (d 4 to hatch)

			Treatment		
Culture system	Incubation period	Chick status	Saran Wrap	Handi Wrap	Intact eggs
		Surviving			
System II	d 0–3	n	42	40	59
		%	97.5[a]	92.4[a]	94.9[a]
		Surviving			
System III	d 4–11	n	41	37	56
		%	75.6[b]	97.3[a]	98.2[a]
		Surviving			
	d 12–18	n	31	36	55
		%	100.0[a]	100.0[a]	100.0[a]
		Hatching			
	d 19–hatching	n	31	36	55
		%	61.3[a]	83.3[a]	92.7[a]
		Hatching			
Overall	d 0–hatching	n	42	40	59
		%	42.9[b]	75.0[a]	86.4[a]

[a,b]Values with different superscipt are significantly different ($P < 0.05$).

not adversely affect the embryos. Between d 12 and 18, embryonic death was not observed in either group. Between d 18 and hatch, the number of embryos that failed to hatch was not significantly different between those sealed with Saran Wrap and Handi Wrap. The lack of statistically significant differences may be related to the number of observations. Peak mortality of eggshells sealed with Saran Wrap occurred between d 5 and 8 of incubation (data not shown), which may be a reflection of the low gas permeability of Saran Wrap that possibly inhibits normal embryonic metabolism. Almost all of the dead embryos were associated with failure to retract the yolk sac and penetration of the chorioallantoic membrane. Over the entire incubation period (d 0 to hatching), embryos cultured with Saran Wrap hatched less compared with embryos cultured with Handi Wrap or control groups. All embryos in surrogate eggshells hatched at approximately d 21.5 compared with d 21 for control embryos.

Table 5 shows the effects of cling-film types on weights of 1-d-old chicks. No significant difference in chick weight was detected between cling-film types. However, the chick weights from surrogate eggshells were significantly smaller than those from intact eggs. Chicks hatching from surrogate eggshell culture were about 4 g less than those from the control eggs. The lower weight ($P < 0.05$) for chicks emerging from the surrogate eggshell cultures was not the result of water loss during incubation because no differences ($P > 0.05$) were found in water loss among the 3 treatments (data not shown). The lower chick weight at hatch could have resulted from embryonic manipulations resulting in increased nutrient use early in incubation to compensate for demands imposed by embryonic manipulation. Despite the lower weight, chicks hatching from surrogate eggshells were normal and healthy and were grown to sexual maturity.

Hatchability of chick embryos from freshly laid eggs cultured in surrogate eggshells in this study was higher than eggs with simple windows (Speksnijder and Ivarie, 2000; Andatch et al., 2004) even though the window opening for surrogate eggshell cultures was larger than traditionally used (Speksnijder and Ivarie, 2000). The large opening of the surrogate eggshell system allowed maximal accessibility to the embryo for manipulation. Furthermore, the development of embryos cultured in surrogate eggshells could be observed continuously throughout incubation. Multiple manipulations at different stages of embryo development could also be performed in surrogate eggshell cultures. Thus, the excellent survival and hatching of chick embryos from freshly laid eggs achieved in this study will promote efficient production of transgenic chickens.

TABLE 5. The effect of surrogate eggshell culture and cling-film types on weights of 1-d-old chicks

Treatment	n	Chick weight
Saran Wrap	13	36.78 ± 0.47[a]
Handi Wrap	20	36.21 ± 0.58[a]
Control group (Intact eggs)	27	40.25 ± 0.40[b]

[a,b]Values represent mean ± SE. Values within column with a different superscript differ significantly ($P < 0.05$). Means are a random sample of the data presented in Table 4.

[1]Dremel, Racine, WI .

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