

《Research Note》

## A Novel Shell-less Culture System for Chick Embryos Using a Plastic Film as Culture Vessels

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The development of shell-less culture methods for bird embryos with high hatchability would be useful for the efficient generation of transgenic chickens, embryo manipulations, tissue engineering, and basic studies in regenerative medicine. To date, studies of culture methods for bird embryos include the whole embryo culture using narrow windowed eggshells, surrogate eggshells, and an artificial vessel using a gas-permeable membrane. However, there are no reports achieving high hatchability of >50% using completely artificial vessels. To establish a simple method for culturing chick embryos with high hatchability, we examined various culture conditions, including methods for calcium supplementation and oxygen aeration. In the embryo cultures where the embryos were transferred to the culture vessel after 55–56 h incubation, more than 90% of embryos survived until day 17 when a polymethylpentene film was used as a culture vessel with calcium lactate and distilled water supplementations. The aeration of pure oxygen to the surviving embryos from day 17 yielded a hatchability of 57.1% (8 out of 14). Thus, we successfully achieved a high hatchability with this method in chicken embryo culture using an artificial vessel.

**Key words:** artificial vessel, chicken embryo culture, hatching, shell-less, polymethylpentene

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### Introduction

A shell-less culture, where a chick embryo is taken from an eggshell and cultured in an artificial environment, is an important technique for the generation of transgenic chickens that produce useful substances in their eggs as well as for various embryonic manipulations (Kamihira *et al.*, 2005; Kyogoku *et al.*, 2008). In addition, this technique could be helpful for the preservation of rare birds, if it can be applied to saving damaged eggs. However, the windowing of an eggshell to facilitate gene introduction and other manipulations significantly reduces the hatchability (Andacht *et al.*, 2004). The shell-less culture technique for chick embryos also potentially plays an important role in the education of school children in life sciences, through the direct observation of embryonic development.

Perry (1988) reported a culture method for chick embryos using a surrogate eggshell as a culture vessel for the *ex ovo* culture of a single-cell stage fertilized egg taken from the maternal oviduct. This method is comprised of three systems: system I for embryos from the single-cell stage to the blastoderm stage, system II for embryos after the blas-

toderm stage until day 3, and system III for embryos from day 3 until hatching. The embryo was cultured in the three systems and transferred from one vessel to another. A large chicken eggshell was used as the culture vessel in system III. However, the hatchability was approximately 7% with this method, which was improved to approximately 50% by Naito *et al.* (1990). Some culture methods using surrogate eggshells have been reported for quails (Ono *et al.*, 1994; Kamihira *et al.*, 1998; Ono *et al.*, 2005; Kato *et al.*, 2013).

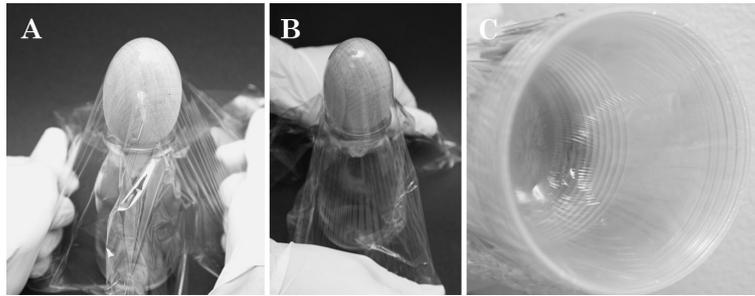
Kamihira *et al.* (1998) reported a shell-less culture method for quail embryos using an artificial vessel made of a gas-permeable polytetrafluoroethylene (PTFE) membrane. In their method, more than 43% quail embryos hatched when calcium lactate was supplemented in the culture. This system corresponded to the Perry's system III and the culture method was also applied to chick embryos (Kamihira *et al.*, 2004).

Various improvements in the culture of chick embryos have been reported. However, many of these methods involved the replacement of surrogate eggshells derived from different bird species laying relatively large eggs, such as aigamo duck and turkey (Borwornpinyo *et al.*, 2005). However, culture methods using surrogate eggshells bear disadvantages such as the preparation of eggshells, differences among batches of eggshells, the inability of recycle use, and the low operability during embryo manipulation.

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**Fig. 1. Processing of the polymethylpentene film.** To prepare a culture vessel, the right and left sides of an approximately 30 cm square film were held and stretched into the appropriate form (A). Further, the sides that remained slack were held and the film was stretched in the same way (B). An oval form was created so as to be approximately the same size as an egg. The film should then be aspirated using an aspirator equipped with a plastic cup, which has the same diameter as the artificial vessel. This also prevented film wrinkling when it was installed on the artificial culture vessel (C).

To establish a simple culture method with a high hatchability, we developed an artificial vessel using a highly convenient transparent plastic film instead of surrogate eggshells, and we examined culture conditions such as calcium and water supplementations and oxygen aeration.

### Materials and Methods

#### Chicken Eggs

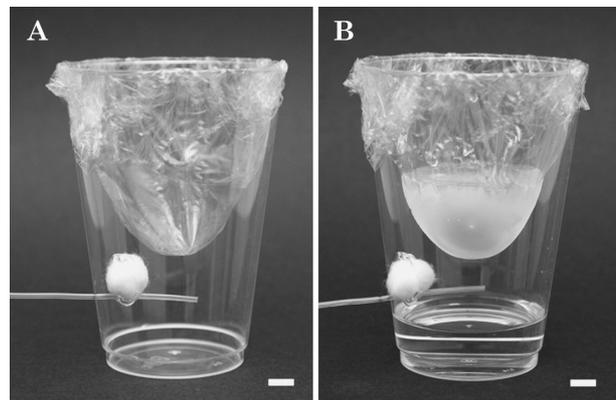
All of the fertilized eggs used in this study were Dekalb Brown eggs, which were obtained from a grocery store (Yamagishism Jikkenchi, Mie, Japan).

#### Culture Vessels

A 430 ml polystyrene plastic cup was used as the pod for the culture vessel. A 1–1.5 cm diameter hole was made in the side of the cup approximately 2 cm from the bottom, and the hole was plugged with a cotton pledget as a filter. A 2 mm diameter plastic tube (Atom Multipurpose Tube; Atom Medical, Tokyo, Japan) was inserted through the space between the pledget and the hole to provide an oxygen supply. An aqueous solution (40 ml) of 0.01% benzalkonium chloride (OSUBAN-S; Nihon Pharmaceutical, Tokyo, Japan; diluted with distilled water) was then added to the cup. A polymethylpentene film (FOR-WRAP; Riken Technos, Tokyo, Japan) was formed into a concave shape, carefully avoiding wrinkles (Fig. 1) and installed as an artificial culture vessel in the pod (Figs. 2A and 3). A polystyrene plastic cover was placed on top of the culture vessel.

#### Embryo Culture

Fertilized chicken eggs were preincubated for 48–50 h (Group A) or 55–56 h (Group B) at 38°C and 60% humidity in an incubator (BITEC-300; Shimadzu RIKKA Co., Tokyo, Japan). For the preparation of embryo culture, 250–300 mg calcium lactate pentahydrate powder (Showa Chemical Co., Tokyo, Japan) was added to the culture vessels. Subsequently, 2.5–3 ml sterilized distilled water (Otsuka Pharmaceutical Co., Tokyo, Japan) was gently added to the



**Fig. 2. Artificial culture vessel used for chicken embryo cultures.** In this figure, benzalkonium chloride was not added (before embryo transfer; A). The artificial culture vessel for chickens (immediately after the embryo was transferred; B). Bar, 1 cm.

culture vessels. Each eggshell was wiped using 70% ethanol, cracked without using a drill, and the whole egg contents were transferred to the culture vessel (Fig. 2B). If broken pieces of the eggshell dropped inside the culture vessel, the pieces were removed carefully using sterilized forceps.

Furthermore, we made 10 ventilation holes with a diameter of 5–8 mm on the upper surface of the polymethylpentene film, with which the embryos did not make direct contact. The holes were created by melting the film using a heated glass rod.

The culture vessel was covered with a polystyrene plastic lid so that the humidity inside the vessel remained at approximately 100%. The culture vessel was maintained at 38°C and 80% humidity in an incubator. The culture vessel was placed with an angle of approximately 8° and rotated

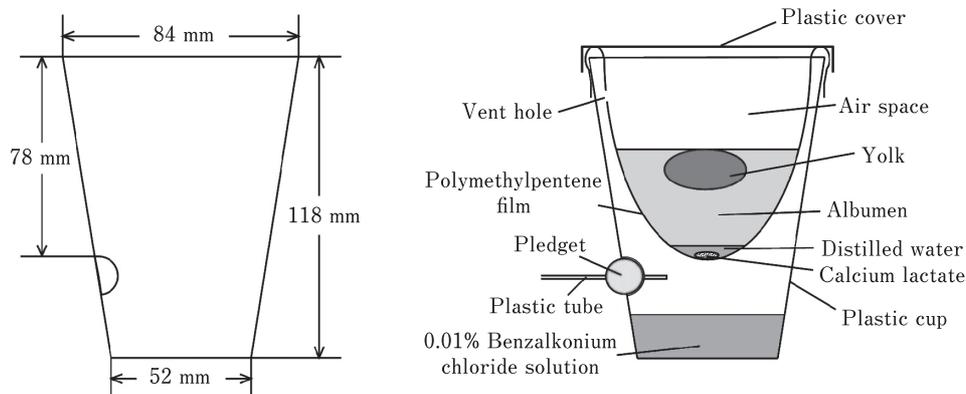


Fig. 3. Schematic drawings of the artificial culture vessel used for chicken embryo culture.

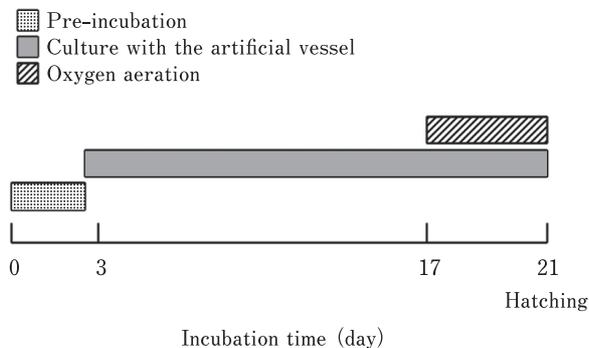


Fig. 4. Culture schedule for chick embryos.

with 120° clockwise, twice a day.

From day 17 of the culture period until hatching, pure oxygen was supplied at a flow rate of approximately 500 ml/h through the previously installed plastic tube.

If the embryo could not rupture the chorioallantoic membrane by itself on days 19 or 20 of the culture period, we carefully created a 5-mm incision in the membrane around the beak without serious bleeding in the membrane. Fig. 4 shows the schedule for the chicken embryo culture. Intact fertilized eggs without manipulation were incubated as controls ( $n=10$ ). They were incubated at 38°C and 80% humidity in an incubator, and rotated with 120° clockwise, twice a day until day 18.

Chickens were given to the new owners and bred to sexual maturation as companion animals.

#### Statistical Analysis

The viability of cultured embryos during the incubation period up to day 17 of the culture was determined by counting the viable embryos, where 100% viability was defined as the viable embryo number on the day at the beginning of the *ex ovo* culture. The hatchability in the presence or absence of oxygen was determined by counting hatched embryos, where the embryo number on day 17, at the beginning of the aeration, was defined as 100%. Data were

analyzed using Fisher's exact probability test. A  $P$  value of  $<0.01$  was considered statistically significant.

## Results

### Preincubation Periods

For the embryos that were transferred to the culture vessel before 50 h incubation (Group A; Stage 12–15; Hamburger and Hamilton, 1951), no embryos survived until day 8. When the embryos were transferred to the culture vessel after 55–56 h incubation (Group B; Stage 16), the viability on day 8 of the culture period was significantly higher than Group A ( $P<0.01$ ; Table 1, Fig. 5), and the viability on day 17 of the culture period was 92% (23 out of 25).

### Oxygen Supply

During the culture in the artificial vessel on approximately day 17 of the culture period (Stage 43–44), the color tone of blood stream in the veins of chorioallantoic membrane changed to dark red, suggesting a deficiency in oxygen supply for the embryos. Therefore, pure oxygen was aerated at approximately 500 ml/h through the plastic tube inserted into the artificial vessel. As a result, in Group B, 57.1% (8 out of 14) of embryos supplied with oxygen developed to hatch. In contrast, no embryos developed to hatch for the conditions where no oxygen was supplied (Table 1, Fig. 6). Thus, the oxygen aeration significantly increased the hatchability ( $P<0.01$ ).

### Growth of Chicks after Hatching

The chicks that hatched in this method were healthy, and they were bred to sexual maturation. After the chickens were mating, we obtained healthy offspring.

### Intact Egg Control

The hatchability of the intact control embryos was 70% (7 out of 10) and no abnormalities were observed. Two eggs were dead with pipped eggs, and another egg was dead at hatching because of damage in yolk sac.

## Discussion

In the present study, we developed a shell-less culture system for chick embryos using a plastic film as culture

Table 1. Summary for chick embryo cultures using a plastic film as culture vessels

Group	Pre incubation	Oxygen supply*	Age at death	
A	48 h	—	8 d	
	48 h	—	8 d	
	48 h	—	8 d	
	48 h	—	8 d	
	48 h	—	5 d	
	50 h	—	6 d	
	50 h	—	5 d	
	B	55 h	—	8 d
		56 h	—	14 d
		55 h	N	18 d
		55 h	N	18 d
		55 h	N	18 d
		55 h	N	19 d
		55 h	N	19 d
		55 h	N	20 d
		55 h	N	20 d
		55 h	N	21 d
55 h		N	21 d	
55 h		S	18 d	
55 h		S	20 d	
55 h		S	20 d	
55 h		S	21 d	
55 h		S	21 d	
55 h		S	Hatching	
55 h	S	Hatching		
55 h	S	Hatching		
55 h	S	Hatching		
55 h	S	Hatching		
55 h	S	Hatching		
55 h	S	Hatching		
56 h	S	Hatching		

—, not applicable; S, oxygen supplied; N, nontreated.

\*From day 17 of the culture period.

vessels, and we examined the conditions required for embryonic hatching by comparing factors such as the addition of calcium lactate and the presence or absence of an oxygen supply. Instead of a gas-permeable PTFE membrane, we used food wraps, which are inexpensive, easy to process, and easy to obtain as culture vessels. Although we tested several wraps, including polyethylene and polyvinylidene chloride, we preferred to use a polymethylpentene film because of its high oxygen permeability ( $8.0 \times 10^{-10}$  mol/m<sup>2</sup> sPa; data from Riken Technos). It is important to eliminate wrinkles when producing a culture vessel because the presence of film wrinkles leads to lower survival rates (data not shown).

In our study, the embryos were preincubated inside the eggshell until day 3 and the eggshell was then cracked to transfer the embryos to the artificial vessel, corresponding to the Perry's system III. The preincubation period until the transfer of embryos to the culture vessel influenced the viability on culture day 8 as well as the successful embryo

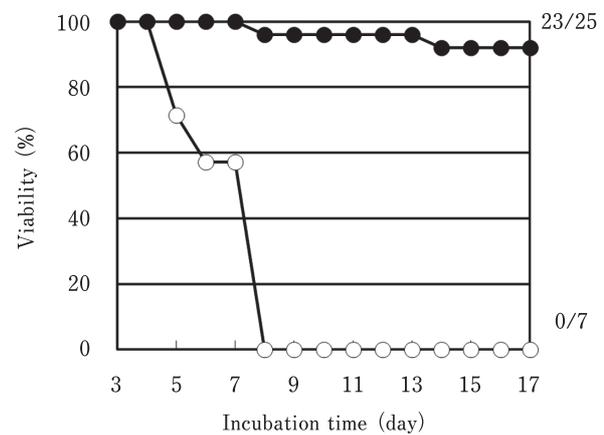


Fig. 5. Effect of preincubation period before transfer on viability of embryos until day 17. Eggs were preincubated for 48–50 h (open circles; Group A) or for 55–56 h (closed circles; Group B). A significant difference in viability on day 8 and 17 between the two groups was observed ( $P < 0.01$ ).

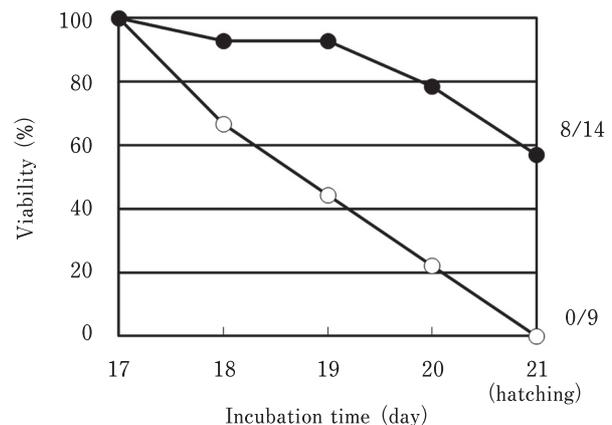


Fig. 6. Viability and hatchability of cultured embryos with or without pure oxygen aeration from day 17. Viability was monitored during the culture period of days 17–21 and the number of hatched embryos was counted. During this period, the embryos were cultured with (closed circles) or without (open circles) oxygen aeration. A significant difference in hatchability on day 21 between with and without oxygen aeration was observed ( $P < 0.01$ ).

transfer to the culture vessel. The transfer of embryos to the culture vessel was considered to be optimal on day 3 (Rowlett and Simkiss, 1987; Borwornpinyo *et al.*, 2005). In the present study, different egg preincubation periods between 48 h and 56 h exhibited variable effects on viability. Significantly higher viability obtained with 55–56 h of preincubation (Stage 16). In our preliminary experiment, the vitelline membrane was often damaged when the embryo was transferred to the culture vessel after 56 h (Stage 17).



Fig. 7. A newly hatched chick from an artificial vessel.

After 60 h, it was difficult to transfer the embryo to the culture vessel by cracking the eggshell because of the risk of damage to the vitelline membrane (data not shown).

A previous study by Kamihira *et al.* (1998) using quails mentioned that calcium lactate was highly effective in successful avian embryo cultures. In our culture system, the addition of calcium lactate was also effective for embryonic development, and the viability of embryos became the highest when 250–300 mg of calcium lactate was added (data not shown). Thus, calcium supplementation to embryos is essential for shell-less cultures, because normal chicken embryos inside eggshells are provided majority of calcium from the eggshell during embryonic development to hatch (Rowlett and Simkiss, 1987; Kamihira *et al.*, 1998). We believe that the amount of calcium lactate supplied to the embryos in the culture was sufficient for the minimum requirement for the embryonic development to hatch, although further studies are still necessary to understand the embryonic availability of calcium.

We were concerned that the aeration of the artificial vessel might cause the moisture loss of embryos by transpiration. Consequently, we first added 2.5–3 ml of sterilized distilled water to the culture vessel. Moreover, when calcium lactate suspended in distilled water was added to the vessel, viability of cultured embryos declined (data not shown), suggesting that this might be caused by an electrolyte imbalance or hypercalcemia. Therefore, we added calcium lactate powder to the culture vessel first, before gently adding distilled water because this might facilitate the slower uptake of calcium lactate by the embryo.

With the culture method used in this study, the veins distributed in the chorioallantoic membrane became dark red at approximately day 17 of the culture period (Stage 43–44), which strongly suggested oxygen deficiency. Rowlett and Simkiss (1989) reported that chick embryos cultured in an

artificial vessel made of a “cling-film” were hypoxic and hypocapnic. Kamihira *et al.* (1998) also pointed out the importance of oxygen supply in the later stage of embryo culture using surrogate eggshell. Therefore, we examined the oxygen aeration in the latter half of the culture period. The oxygen aeration during the initial stage of culture may reduce viability due to the toxic effect of oxygen. In the present study, the color of blood stream in the chorioallantoic membrane appeared to be normal during the initial stages of the culture period. Therefore, for the embryos that survived until day 17 (Stage 43–44), pure oxygen was aerated from day 17 to prevent oxygen deficiency in the later stages. As a result, the color tone of blood stream became normal, and 8 out of 14 embryos developed to hatch, demonstrating that the oxygen supply in the later stage is extremely important during shell-less culture using an artificial vessel.

On days 19–20 of the culture period (Stage 45), the embryos began to tear the chorioallantoic membranes and their pulmonary respiration systems were activated. When the embryos could not tear the chorioallantoic membrane, a 5-mm incision around the beak was made to promote hatching. This may correspond to death in pipped eggs, which occurs at a constant rate when eggs are cultured without shell cracking and treatment.

The results revealed that using the culture vessel described in the present study, the highest hatchability was achieved with 55–56 h of egg preincubation, addition of 250–300 mg calcium lactate, addition of 2.5–3 ml sterilized distilled water, aeration of pure oxygen at approximately 500 ml/h from day 17 of culture (Stage 43–44). The hatchability of embryos cultured under these conditions was 57.1% (8 out of 14) when taking the number of surviving embryos at day 17 as 100%.

Borwornpinyo *et al.* (2005) reported that chicken embryo culture method using turkey eggshells and Handi-Wrap as culture vessels before transitioning to Perry’s System III exhibited 81.1% hatchability when the number of surviving embryos at day 3 was taken as 100%. Although our culture method does not use surrogate eggshells, a high hatchability of more than 50% was achieved. This method does not require any complicated operations, special materials, or techniques. It also eliminates uncertainties such as availability and quality difference of eggshells, which frequently caused problems in previous methods using surrogate eggshells. Furthermore, the use of a transparent plastic film allows not only easy observation of the embryonic morphology from all angles but also easy access to the embryo for manipulation by removing the plastic cover. At present, further improvement of the culture method is being attempted to increase the hatchability.

If we can obtain a stable hatchability, this method could facilitate the generation of transgenic chickens and other embryonic manipulations, which are required for basic studies in regenerative medicine. It could also support tissue culture, studies using avian embryonic stem cells, and the mass production of chicken eggs as living bioreactors.

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