

◀ Review ▶

Ex Ovo Culture System for Avian Embryos and its Application

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Ex ovo culture of avian embryos can be applied not only to embryology but also to various fields of basic research such as embryo manipulation, toxicology, and regenerative medicine. The windowing method, which facilitates various manipulations and observations by opening a hole in one part of the eggshell, and culture systems using surrogate eggshells, are widely used. Despite this, biology lessons in high schools cover shell-less culture systems, which involve the development of avian embryos in artificial vessels, such as rice bowls, without using surrogate eggshells. However, as embryo development stops at its early stages in this method, it is not possible to continuously observe the development of the embryo. This led to attempts to develop an embryo culture method using a complete artificial culture vessel that does not use surrogate eggshells, and Kamihira *et al.* (1998) succeeded in hatching quail embryos in an artificial culture vessel using polytetrafluoroethylene membranes. In addition, Tahara succeeded in hatching chick embryos in artificial culture vessels that used cling film made of polymethylpentene and reported their detailed methodology (Tahara and Obara, 2014). These technologies are being applied not only to school education but also to various fields of research.

Key words: Avian embryo, chicken, hatching, polymethylpentene, shell-less culture system, *ex ovo* culture system

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Introduction

Ex ovo culture of avian embryos*¹ has contributed widely to basic research. These technologies are expected to be applied to various fields, including basic research intended for embryo manipulation experiments, toxicity tests, and regenerative medicine. Biology lessons in high schools also use *ex ovo* culture systems to provide students a better understanding of the process of embryonic development.

Although various studies on the *ex ovo* culture systems of avian embryos have been conducted, it is difficult to establish an *ex ovo* culture system in which it is possible to hatch chicken eggs; thus, a trial and error approach has been used for a long time. Since the first report on hatching in a culture vessel using surrogate eggshells by Rowlett and Simkiss (1987), various improvements have been made. However, methods that use surrogate eggshells have an issue; eggshells

suitable for culturing chick embryos must be prepared and any observations or manipulations of the embryo are limited by the visual limitations imposed by the eggshell. Therefore, a study of a shell-less culture system that hatches embryos completely in artificial vessels*² was conducted and Kamihira *et al.* (1998) reported on their success in hatching quail embryos in a culture vessel that used polytetrafluoroethylene (PTFE) membranes. Following this, Tahara succeeded in hatching chick embryos in completely artificial culture vessels that used food wraps made of polymethylpentene and reported the detailed methods along with consideration for optimum conditions (Tahara and Obara, 2014).

In this review, we will introduce the transitions in the *ex ovo* culture system and shell-less culture system technologies, their advantages and disadvantages, as well as the applications of these technologies.

Ex ovo Culture System

The development of avian embryos after ovulation, unlike that of mammals, take place outside of the body; thus, avian embryos are used in a variety of research fields, including embryology, toxicity tests, and embryo manipulation. In

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¹ *Ex ovo* culture system: Removal of the original eggshell or transfer of the embryo before eggshell formation to a culture vessel for culturing. This includes the case in which a surrogate eggshell is used as the culture vessel.

² Shell-less culture: A method of *ex ovo* culture that does not use a surrogate eggshell.

particular, the windowing method, which opens a hole in one part of the shell to carry out various manipulations, has now been used in many fields of study (Fisher and Schoenwolf, 1983; Andacht *et al.*, 2004).

In the *ex ovo* culture of chicken embryos, Rowlett and Simkiss (1987) removed the eggshell from the fertilized egg on Day 3 of incubation, transferred the egg contents to a culture vessel that used the eggshell of a turkey as a surrogate eggshell, and succeeded in hatching the embryo. However, attempts to hatch the embryo by shell-less culture systems using food wraps (cling film) did not result in success.

Perry (1988) was the first to successfully hatch chick embryos from fertilized eggs at the 1-cell stage using an *ex ovo* culture. In this method, pronuclear stage fertilized eggs removed from the hen's oviduct are hatched using three systems. First, fertilized eggs removed from the hen's oviduct are transferred to a glass vessel, together with the culture solution, and cultured for 24 hours until the blastoderm stage (System 1). These are then transferred to a vessel that uses a surrogate eggshell, filled with thin albumen. The embryos are cultured with the side of the vessel fixed; thus, the embryos do not come into contact with air (System 2). Next, the embryos on day 3 of incubation are transferred to a large surrogate eggshell such as a double-yolk egg, the air layer is formed, and the embryos are cultured until hatching (System 3). The hatching rate by this approach is approximately 7%.

After this, improvements, such as removing albumen and filling with thin albumen when transferring the embryo from System 1 to System 2, were developed, which increased the hatching rate to 30%–50% (Naito and Perry, 1989; Naito *et al.*, 1990).

However, the eggshell, which serves as the vessel, becomes essential in approaches to culturing that use surrogate eggshells. Large-sized eggshells, such as double-yolk eggs, and eggshells of birds that lay eggs that are slightly larger than chickens, such as ducks and turkeys, have been used as surrogate eggshells (Rowlett and Simkiss, 1987; Borwornpinyo *et al.*, 2005).

The establishment of the windowing method has led to its application in various fields of research, including studies in embryology and the mechanism of malformations (Matheus *et al.*, 2019), imaging (Kulesa *et al.*, 2010; Funahashi *et al.*, 2014), embryo manipulations such as xenotransplantation (Boulland *et al.*, 2010), chorioallantoic membrane assays (Ribatti, 2016; Vu *et al.*, 2018), evaluation of pluripotency in stem cells (Haraguchi *et al.*, 2016), and basic research in regenerative medicine (Chiba *et al.*, 2010). However, although methodologies that use the windowing approach and surrogate eggshell approach managed to continue embryonic development after various treatments, it was difficult to observe the processes of development. Furthermore, methods that use surrogate eggshells have difficulties in preparing a large number of surrogate eggshells at once, which led us to think that there is a need to develop a completely artificial culture vessel that does not use eggshells. We also conceived that development of an artificial culture vessel that does not use surrogate eggshells would be a technology that

is needed to analyze the influence of eggshells on the embryo.

Development of an Artificial Culture Vessel using a PTFE Membrane

An artificial culture method that does not use a surrogate eggshell has been in development for a long time and culture methods that used polyethylene bags and cling films were able to develop the embryo continuously in its early stages. However, it is not possible to hatch the embryos this way and various problems, such as breathability and the supply of calcium to embryos, were pointed out (Elliott and Bennett, 1971; Rowlett and Simkiss, 1987).

Kamihira *et al.* (1998) succeeded in hatching quail embryos in an artificial culture vessel using PTFE membranes without using any surrogate eggshells. In this method, eggshells are removed from eggs on day 2 of incubation, the embryos are cultured by adding eggshell powder and calcium lactate to the artificial culture vessel as sources of calcium, and pure oxygen is supplied to the incubator. They reported that 42.8% of the embryos hatched. They also reported that, even in artificial embryo cultures that use surrogate eggshells, the hatching rate increases after the addition of calcium lactate. These results indicated that addition of calcium lactate is important in *ex ovo* culture systems.

Tahara's Development of a Simple Culture Vessel

Although the artificial culture vessel that uses PTFE membranes has the significant advantage of not needing a surrogate eggshell, PTFE membranes are opaque, observation is difficult from directions other than through the top of the vessel, and the PTFE membranes are difficult to obtain. Tahara developed an even simpler artificial culture vessel intended for application in biology laboratory classes in high schools. Using a cling film made of polymethylpentene as a culture vessel, adding calcium lactate as a calcium source to the embryo, and then pre-culturing the embryo for 55 hours (Stage 16; Hamburger and Hamilton, 1951) before transferring the embryo to the culture vessel, continuing the culture, and directly supplying pure oxygen into the culture vessel from day 17, embryos can be successfully hatched (Tahara and Obara, 2014). As this method uses a transparent film as the culture vessel, it not only facilitates the observation of embryonic development but also provides easier access to the embryo than conventional methods. Therefore, this method can be easily applied to various fields of study, such as bio-imaging, embryonic manipulation, and chorioallantoic membrane assays.

Practical Shell-less Culture of Embryos in High Schools

Continuous observation of the developmental processes of avian embryos and ultimately hatching the embryos would be impossible in general facilities provided in Japanese public high schools. High school biology textbooks in Japan introduce approaches that cut the embryo with the vitelline membrane (New, 1955) and shell-less culture systems that



Fig. 1. Chicken embryo on day 11 of culture, based on the method of Tahara and Obara (2014).

use rice bowls as culture vessels to observe the early development of chicken embryos (Motokawa *et al.*, 2008). These approaches have also been demonstrated in class. However, these approaches have not achieved embryo hatching.

For more than 40 years, Tahara and colleagues, have repeated trial and error experiments with high school students, reported repeatedly at the meeting gathered high school biology teachers in Japan, and produced reports with the aim to develop a new method for the continuous observation of development and hatching of avian embryos using a shell-less culture for high school biology classes and biology clubs. These studies have also been introduced in high school biology textbooks in Japan (Hotta *et al.*, 2010). In 2012, Tahara succeeded in hatching chicks for the first time from shell-less culture using cling films at the Chiba Prefectural Oihama High School and reported the experimental procedures in detail with further consideration of optimal conditions (Tahara and Obara, 2014; Tahara, 2016).

Since these reports, Tahara and colleagues have carried out further practical demonstrations in classes and biology clubs to hatch chicks using the technology they developed. The culture vessel used in this method allows observation from various angles because the vessel is transparent. In addition, high school students can make their own culture system and are able to culture embryos until hatching in a non-sterile environment. This method enables high school students to perform these experiments as part of high school lessons (Fig. 1).

Students who wish to participate are asked to raise the chicks after the shell-less culture experiments in their own homes. However, in urban areas, the number of households that can raise chickens is limited due to various factors such as the surrounding environment. Therefore, Tahara examined the conditions to determine whether the chicken shell-less culture approach can be applied to quail, which are easier



Fig. 2. Biology laboratory class at high school using quail eggs.

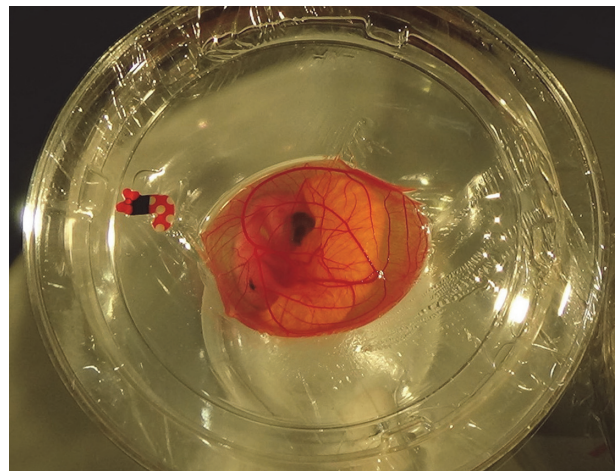


Fig. 3. Quail embryo on day 10 of culture, applying the method of Tahara and Obara (2014).

to raise, and succeeded in hatching quail embryos. In recent years, the Chiba Prefectural Oihama High School has been using quail shell-less culture for experimental demonstrations in class (Figs. 2 and 3).

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Conflicts of Interest

The authors declare no conflict of interest.

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