A New Technique for Explantation and In Vitro Cultivation of Chicken Embryos

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ABSTRACT A technique is described for explanting and cultivating chicken embryos in plastic drinking cups which have been modified with plastic wrap to reproduce the geometry and dimensions of the egg shell. Successful explantation rates of 97% are possible with a double-window technique, and survivability in cups exceeds that achievable in other in vitro systems (i.e., petri dishes). Long-term survival to the 21st day of incubation is seen routinely. This system with cups is less expensive than that with petri dishes, and simpler than that with plastic wrap/tripods. Thus, this new method of in vitro cultivation of chicken embryos improves upon explantation rate, survivability and system design, and has a wide range of applications in developmental biology, angiogenesis, cancer, and pharmacology research.

Shell-less culture of chicken embryos enables a more thorough analysis of embryonic development and physiology since it allows continuous observation and access to the embryo (Dunn et al., 1981; Ausprunk et al., 1974). In addition, the vascularized membranes of the chick embryo, which include the yolk sac membrane and chorioallantoic membrane (CAM), provide useful models for studying angiogenesis (Folkman, 1974a,b, 1985) and bioassays that aided the isolation and identification of several angiogenesis factors (Folkman and Klagsbrun, 1987). It is ideal for studying the vascular response to tumors, as the immunoincompetent chick tolerates a variety of implanted tumor grafts without rejection (Folkman, 1974b; Brem and Folkman, 1975). Chick embryo models are conducive to investigations of pharmacologic agents and their mechanisms of action, since these assays are rapid, inexpensive, and allow localized application of drug and large sample sizes. There are myriad other uses for the chick embryo in a variety of research fields, making it a potentially widespread research tool.

The advantages of chick embryo models have been offset by difficulties in establishing simple, reliable, cost- and time-effective techniques. Egg-shell windows are tedious to make, often induce inflammation in the CAM (Folkman et al., 1983), and are too small to adequately visualize activity. Explantation, i.e., transfer of the embryo from its egg shell to an in vitro system, solves many of these problems (Auerbach et al., 1974), but existing techniques with the petri dish system are expensive and long-term survivability has been low. A plastic wrap/culture tripod system has been introduced with excellent survival results (Dunn et al., 1981), however the tripod construction is complex, which may limit its widespread use.

We have developed new approaches to shell-less culture of chick embryos which include 1) a new double-window technique for embryo transfer and 2) an in vitro system which uses plastic wrap to mimic natural egg contours, but uses plastic cups instead of tripods to simplify the apparatus. We believe that improved techniques and simple systems increase long-term survivability of embryos in shell-less culture and make these models less expensive and more practical.

MATERIALS AND METHODS

Plastic-Wrap Hammock Preparation

Clear plastic 7 ounce drinking cups (Solo Company, P-7A) are filled with approximately 30 ml water for stabilization. Plastic wrap (Saran Wrap, Dow Chemical Company) is used to form a 4 cm-deep concave well in the cup, and its edges are secured with a rubber band. The extra edges of the plastic wrap are trimmed away and the cup is capped with an air-tight lid (Solo Company, No. 57TL). The cups are then sterilized with 1–2 hours of ultraviolet radiation, after which they are ready for explantation.

Explantation With Double-Window Technique

Four day-old fertilized White Leghorn chicken eggs are obtained (Truslow Farms Inc., Chestertown, MD) and wiped clean first with 70% ethanol and then povidone-iodine solution (Betadine® Solution, Purdue Frederick Company, Norwalk, Connecticut), and allowed to air-dry with the wide end down. The air sac is located at this end and when held in this orientation the embryo floats to the top, away from subsequent manipulations. The egg is cracked over the wide end and the air space is exposed to its edges by removing shell fragments and outer shell membrane with forceps (Fig. 1). This opens a window through which the egg

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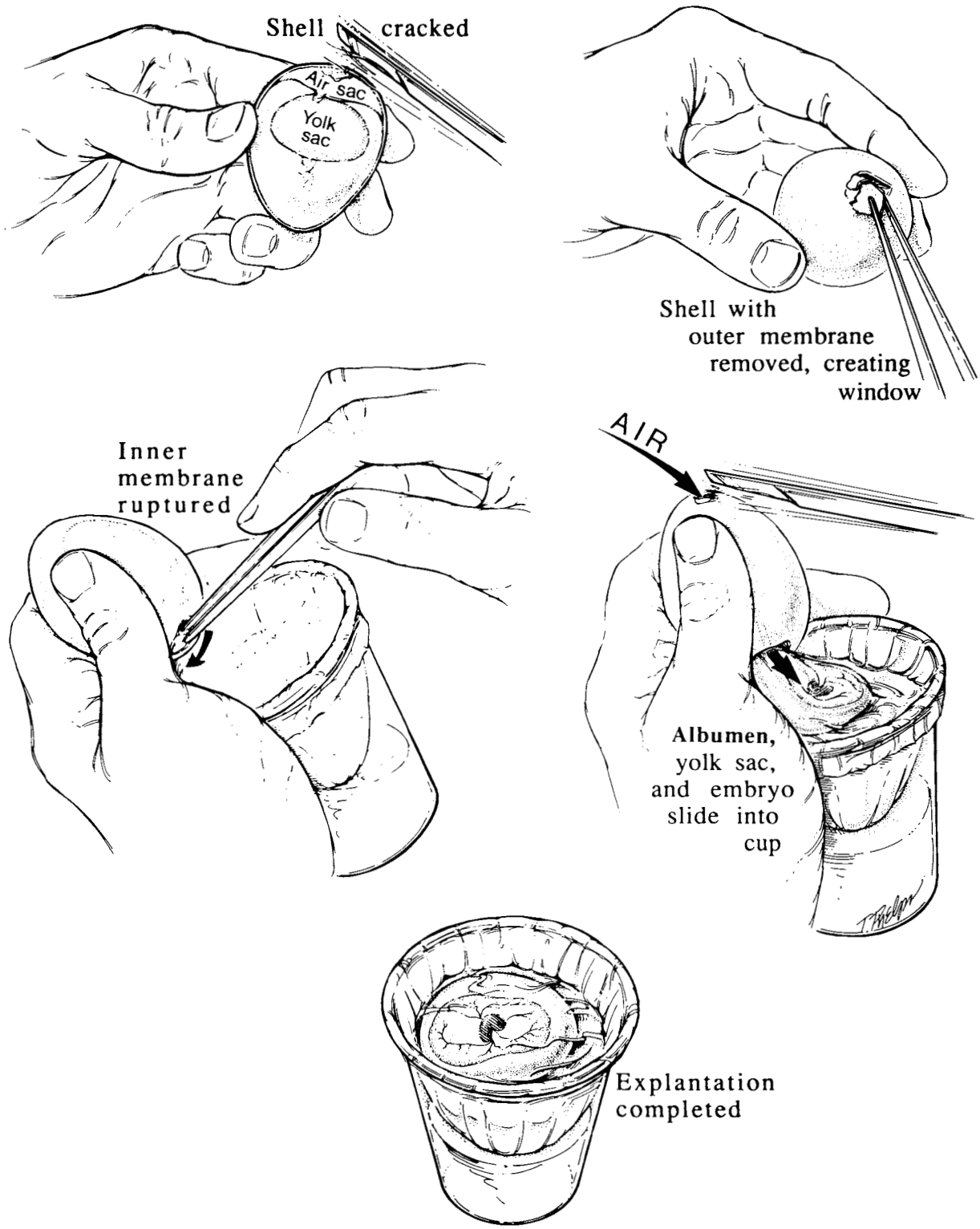

Fig. 1. The double-window technique for explantation of chick embryos. See text for full descriptions of technique and apparatus.

contents can be visualized and, later, will pass. Sharp edges around the window are removed carefully.

The inner shell membrane is pierced or pulled outward with a forceps so that its concave surface becomes convex. This manipulation transfers the air space to the opposite, apical end, above the embryo. A small

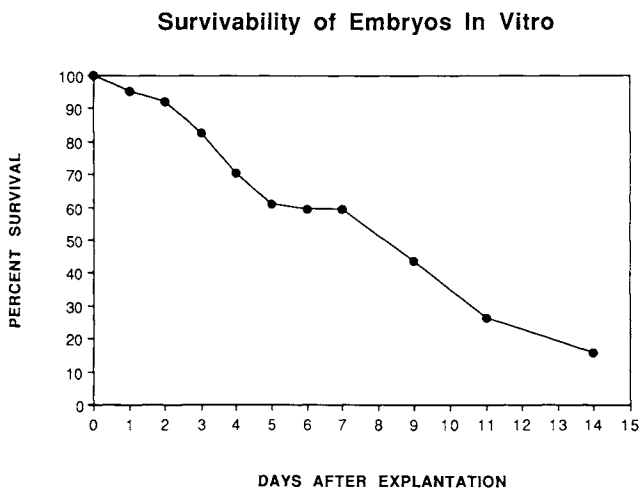

Fig. 2. Survivability of chick embryos in the plastic wrap/cup apparatus. Embryos were transferred after 4.5 days of pre-culture incubation. The N value equals 64.

amount of albumen may be released, but the egg contents should remain in the shell since there is still a vacuum in the apical space.

The egg is then held over the cup with the opened window facing downward to allow the embryo and yolk to float to the top. The cup is held with the same hand as the egg, and together they are tilted off the vertical. The egg contents are released by cracking the apical end of the egg with a blunt instrument, thereby creating a second window in the shell which allows air to enter the apical space and remove the vacuum. The albumen, yolk sac, and embryo slide into the cup through the bottom window, and the transfer is completed. Occasionally the contents are not released spontaneously, and in this case rotation of the egg along its long axis will free the contents. The cups are capped with their lids and placed in an egg incubator (Model no. 50, Humidaire Incubator Company, New Madison, OH) at 37°C with 60% relative humidity.

RESULTS

Explantation

This technique enables a 97% rate of successful transfer of embryos. Optimum transfer time is at day 4.5 of development. Chicks explanted before 4 days have a significantly reduced survival rate (10% at 6 days with 3.5 day-old chicks). In contrast, eggs attempted after 4.5 days become increasingly difficult to explant with time as their membranes become more adherent to the shell membrane and susceptible to the trauma of the procedure. Day 4.5 optimizes developmental maturity with ease of explantation. Preparation of cups and explantation of 100 eggs can be done by an experienced person in 3.5 hours.

Survival

A survival curve for explanted eggs is plotted (N = 64 eggs), showing a loss of approximately 7% per day throughout (Fig. 2). Eighty three percent of the embryos are alive after 3 days of cultivation, and 50% are alive after 8 days. Normal chicks hatch on the 20th–21st day (day 16 in culture). The longest survivor in the cup lived 22 days, reaching the Hamburger-Hamilton stage 41 (Hamburger and Hamilton, 1951). This suggests some developmental delay similar to that reported with explanted chicks (Auerbach et al., 1974).

DISCUSSION

The techniques of chick embryo models have undergone an evolution, from using egg-shell windows to explantation into petri dishes to explantation into contoured plastic wrap. Creating egg-shell windows is a time-consuming process with considerable morbidity and poor visualization, making quantification of the results difficult. Explantation is rapid and enables excellent visibility, but carries with it a significant decline in survivability when flat dishes are used. Plastic wrap systems mimic the egg shell shape in vitro to combine the beneficial features of explantation and the natural egg contour, representing a further evolution of technique. With our modifications in technique and construction, plastic wrap systems should improve embryo survival and enable faster, easier application of these models.

In contrast to the petri dish, the plastic wrap/cup apparatus reproduces both the geometry and dimensions of the egg shell. The average egg length is 6 cm, and the cup diameter is 6.5 cm. This diameter is in accord with the optimal diameter for embryonic survival and growth reported by Dunn et al. (1981). The average egg width is 4.5 cm, and the depth of the hammock is made to match this. When the egg contents rest in the cup, the yolk is suspended in albumin and the embryo rests on top of it. As the CAM develops and enlarges, it rises to the liquid-air interface as a functionally intact, smooth plane of vessels with excellent visibility and accessibility.

Integrity of the yolk sac is critical for survival. The suspended yolk can conform to its most energetically stable shape, a sphere. Since a sphere has the smallest surface area for a given volume, then by Laplace's law the sphere minimizes surface tension in the yolk sac membrane. In both the natural egg shell and the cup technique, the yolk assumes this spherical shape with minimal surface tension. In contrast, the yolk is deformed as it sits in a petri dish and its membrane must withstand a higher surface tension. Small leaks are observed in the yolk sac membrane at 24 hours in the petri dishes that were not present initially (Auerbach et al., 1974), perhaps attributable to an increased rate of membrane breakdown. Survival in cups is slightly higher than in petri dishes. After 3 days of cultivation, 83% are alive in cups as compared to 71% in petri dishes (Crum et al., 1985). By day 5, survival is 70% in cups vs. 68% in petri dishes (Crum et al., 1985). The increased survival of the embryo in cups vs. petri dishes may reflect the difference in surface tension of the yolk sac membrane imposed on it by its container.

Our double-window technique of embryo transfer accelerates this tedious task without sacrificing embryo safety. The "C" clamp technique, which involves carefully cracking the shell with controlled pressure and enlarging the crack with forceps (Dunn et al., 1981), is slower than either the "fried-egg" or double-window

techniques. Greater transfer survival with the double-window (97%) vs. the fried-egg technique (80–87%) suggests that it is less traumatic (Auerbach et al., 1974; Crum et al., 1985). Transfer of embryos with our method can be delayed until much later in embryo life, which is advantageous since increasing pre-culture incubation significantly improves embryo survival (Dunn et al., 1981). Difficulties transferring embryos with other techniques have prevented pre-culture incubation beyond 3 days, and both Auerbach et al. (1974) and Dunn et al. (1981) report this as their optimal transfer times. The double-window technique enables pre-culture incubation to 4.5 days which may increase survivability with this method.

We introduce the use of cups to support the plastic wrap hammock as a modification of the tripod apparatus. This modification makes plastic wrap systems easy to assemble, standardized in dimension, disposable, and reproducible. The brand of plastic wrap used to make the hammock is an important variable of the apparatus. The permeability of plastic wrap to gas exchange and the surface characteristics of the material vary between brands and have an established effect on survivability of embryos (Dunn et al., 1981). In our experiments we used Saran wrap and obtained comparable survival rates to tripods which use Saran wrap (Dunn et al., 1981).

We have presented a new technique for explantation and cultivation of chick embryos that improves shell-less culture techniques in terms of transfer rates and survivability. Perhaps with this technique the many advantages of chick embryo models can be utilized more effectively in research and with greater ease for researchers. We feel that the CAM assay provides an excellent in vivo system for studying angiogenesis activity and the effects of biologic response modifiers, as it is well-suited to rapidly testing therapeutic agents in large quantities and manipulating multiple experimental variables. It is our hope that these new technique will assist those attempting to develop therapeutic modalities in angiogenic diseases (Folkman and Ingber, 1987; Folkman, 1989) as well as those in the field of developmental biology searching for better embryonic models.

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