A scanning electron microscope study of the hatching of bovine blastocysts *in vitro*

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Summary. The blastocysts were recovered from cows 7–10 days after oestrus and cultured. The zona pellucida has a spongy fibrous structure. Hatching begins, about 24 h after culture, through a relatively small hole out of which the blastocyst appears to escape by its own activity. Later the zona becomes split and the two edges of the slit surround part of the blastocyst. The emerging cells were large or small blastomeres which were generally covered by a dense mass of microvilli.

Introduction

As a criterion for testing cow blastocysts for their short-term developmental potentialities, we have used their ability to hatch *in vitro*. Whether the zona has been shed can be ascertained rapidly and it is a much more clear-cut assessment than, for example, increase in size. Furthermore, the number of blastocysts hatching is a measure of the adequacy of our culture conditions (Renard, du Mesnil du Buisson, Wintenberger-Torres & Ménézo, 1976).

In the present study we examined mainly the ultrastructure of cultured blastocysts that were just beginning to hatch. The aim of the study was to describe the process of hatching of cow blastocysts as observed *in vitro* and to discuss the possible way(s) of zona shedding *in vivo*.

Materials and Methods

Blastocysts were obtained from cross-bred Charolais heifers. Superovulation was induced by the injection of 2100 i.u. PMSG between Days 9 and 11 of the cycle (Day 0 = day of oestrus) followed 48 h later by an injection of 500 µg Cloprostenol (ICI 80,996), a prostaglandin analogue. The animals were then inseminated twice with frozen semen at the time of oestrus, which was detected by testing with a vasectomized bull twice daily.

The heifers were slaughtered on Days 7–10 of the induced cycle and embryos were recovered by perfusion of the uterine horns with phosphate-buffered saline (290 mosmol; pH 7.2–7.4). Well developed blastocysts were selected with a stereomicroscope at $\times 40$ magnification. The diameters of the zona and of the vitellus of each blastocyst were measured with an ocular micrometer. More than 10 blastocysts at different stages of hatching and a few empty zonae pellucidae were prepared for scanning electron microscopy either directly after recovery or after culture for 24 to 72 h at 37°C in 0.5 ml B2 medium (Ménézo, 1976) under a gas mixture of 90% N₂, 5% O₂, 5% CO₂, conditions which gave hatching of 31 out of a total of 47 blastocysts collected between Days 7 and 9.

The blastocysts were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.3) and stored in the fixative. After washing in the cacodylate buffer, they were post-fixed in 1% osmium tetroxide and then subjected to critical-point drying in Freon or CO₂, as described by Fléchon, Bergström, Jaszczak & Hafez (1975). Some of the embryos were processed in carriers enclosed by grids of the standard type used for transmission electron microscopy (Taylor, 1975). Specimens were coated with carbon and gold–palladium and were observed at 15 or 20 kV.

Results

Zona pellucida structure

The zona pellucida of the 7–10 day unhatched cow blastocyst appeared as a slightly rough continuous envelope (Pl. 1, Fig. 1). At higher magnification its structure appeared to be porous, at least in surface view (Pl. 1, Fig. 2).

The structure of the zona round embryos hatching *in vitro* did not appear to have changed significantly (Pl. 1, Figs 3 and 4). After hatching *in vitro* or *in vivo* the zona retained its original shape, and a slit, about half the perimeter long, was visible (Pl. 1, Fig. 5). In the angle formed by the edges of the slit through which the blastocyst had escaped, a fibrous configuration was observed within the zona (Pl. 1, Fig. 6). Variable amounts of contaminant material were present on the zona before, during and after hatching (Pl. 1, Figs 1–6).

On Days 9 and 10, the mean $\pm$ s.e.m. diameter of the zona of the unhatched blastocysts was $193.5 \pm 28.4 \mu\text{m}$ ($n = 21$). On the same days, the diameter of the vitellus of the hatched blastocysts was $221.8 \pm 42.5 \mu\text{m}$ ($n = 17$). Immediately after recovery, collapsed blastocysts were frequently observed inside a split zona. The mean $\pm$ s.e.m. diameters of the vitellus and zona of these blastocysts were respectively $164.7 \pm 15.8 \mu\text{m}$ and $188.4 \pm 16.7 \mu\text{m}$ ($n = 19$). Such blastocysts were not degenerating because they expanded *in vitro*.

Blastocyst hatching

The first blastomeres appeared through a hole in the zona pellucida and were of two kinds, small round cells and larger flattened epithelial-like cells (Pl. 2, Figs 7 and 8). Most of these cells (with only a few exceptions) were covered with a large number of microvilli (Pl. 2, Figs 7 and 8). The older blastocysts escaping through a larger split in the zona appeared slightly squeezed by the two edges of the slit (Pl. 2, Fig. 9). The trophoblast had a fairly uniform appearance and consisted of flat epithelioid cells. Nevertheless, it was possible to observe cells of different shapes and size, some large and flat, some slightly dome-shaped and some very small (Pl. 2, Figs 10 and 11). Sometimes, small round cells were present on the surface of blastocysts (Pl. 2, Fig. 9).

In blastocysts of 10 days or older (after culture *in vitro*), parts of the surface of some of the cells had few microvilli, for example, where the surface was slightly convex (Pl. 2, Figs 10 and 11). However, most blastomeres were uniformly covered with a dense mass of microvilli, amongst which a few cytoplasmic blebs were occasionally seen (Pl. 2, Fig. 12).

Discussion

The zona pellucida of the cow blastocyst appears to be viscous or adhesive because contaminants were seen on its surface, although they may have been precipitated during fixation or dehydration; it has a fibrillar spongy structure similar to that which has been observed by transmission and scanning electron microscopy of the eggs of other species (Zamboni, 1971; Fléchon *et al.*, 1975; Dudkiewicz, Shivers & Williams, 1976; Dudkiewicz, Williams & Humphreys, 1976). Although the zona of mammalian eggs is known to consist of glycoproteins (Fléchon, 1970, 1974; Nicolson, Yanagimachi & Yanagimachi, 1975) and to be digested by proteolytic enzymes and agents which split the disulphide bond (Inoué & Wolf, 1974), the structure of the bovine zona was not profoundly changed during or after hatching; there was no uniform lysis, only perforation in one place.

The time of hatching of superovulated eggs *in vitro*, even after 3 or 4 days in culture, corresponds approximately with the time of zona loss *in vivo* (9–10 days after oestrus; Renard *et al.*, 1976). As we were unable to collect blastocysts during the process of hatching *in vivo*, it is difficult to say whether the shedding of the zona *in vivo* was similar to that observed *in vitro*. However, the appearance of a zona pellucida from which a blastocyst had recently hatched at the time of flushing (Pl. 1, Fig. 5) supports this hypothesis; furthermore, empty zonae with equatorial slits were often found in the uterus *in vivo*

at least until Day 13 after oestrus, a long period after the normal time of zona shedding. Although flushing the uterus may rupture the zona pellucida, our observations suggest that the process of hatching in the cow is the same *in vivo* as *in vitro*.

Comparison of the events described with those in the rabbit, a species in which dissolution of the egg envelope has been well studied (Denker & Hafez, 1975), is difficult because the preimplantation rabbit blastocyst is surrounded by a mucin coat (Enders, 1971). In rats and mice the blastocyst may play an active role in hatching (Bitton-Casimiri, Brun & Psychoyos, 1970; Bergström, 1972), or the uterus may be the principal site of activity (Mintz, 1970; McLaren, 1970). The zona pellucida seems to be torn by the hatching cow blastocyst, as indicated by the stretched fibres seen in the angle between the edges of the slit. Our observations on the diameter of blastocysts suggest that expansion and contractile activity may induce hatching; the diameter of the zona pellucida effectively increases because at the fertilized egg or early cleavage stages it is $\leq 160 \mu\text{m}$. Unlike Wright, Anderson, Cupps & Drost (1976), we did not measure the thickness of the zona; we would ascribe an eventual thinning of the zona before hatching to distension by the blastocyst rather than to lysis.

There has been no previous ultrastructural study of the early preimplantation embryo of the cow, as there has been for the ewe (Wintenberger-Torres & Fléchon, 1974; Calarco & McLaren, 1976). The small and round blastomeres of the 7- to 9-day-old cow blastocyst are similar to those described for baboon morulae (Fléchon, Panigel, Kraemer, Karter & Hafez, 1976; Fléchon, Kraemer & Hafez, 1977). Polar bodies may also be present even after hatching. In the 9- to 10-day-old blastocysts most of the surface blastomeres looked like the large ones of the younger embryos, although differences of size and shape still existed. In spite of a few exceptions the microvillous covering of the cow blastocyst appears very uniform, in contrast to that, for example, of the rabbit (Fléchon *et al.*, 1975).

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1.** General aspect of the zona of an unhatched 10-day cow blastocyst.
- Fig. 2.** Higher magnification showing the spongy structure of the surface of the zona.
- Fig. 3.** General aspect of the zona of a hatching blastocyst, cultured *in vitro* from Day 9 to Day 10.
- Fig. 4.** Higher magnification of the same zona. Comparable view to that shown in Fig. 2.
- Fig. 5.** Empty zona after early hatching *in vivo* (Day 7); an extruded blastocyst was found at the same time. Note the retention of the spherical shape and the open slit.
- Fig. 6.** The angle between the two edges of the zona slit stretched by a hatching blastocyst cultured from Day 9½ to Day 10½. The inner part of the zona has a fibrillar structure. The surface of a blastomere is visible in the lower half of the picture.

PLATE 2

- Fig. 7.** Early phase of hatching through a small hole of the zona round a blastocyst cultured from Day 7 to Day 9.
- Fig. 8.** Detail of the extruded cell mass of the same blastocyst: large and small blastomeres are segregated and all are covered by microvilli, except for the blastomere(s) on the top of the cell mass which have no microvilli, only a few blebs.
- Fig. 9.** Blastocyst almost completely extruded from a half-opened zona after culture from Day 9½ to Day 10½. Small round cells are visible on the surface of the blastocyst which looks larger in diameter than the zona.
- Fig. 10.** Higher magnification of the surface of the blastocyst close to the zona (top): the blastomeres are generally large and uniformly covered by microvilli, with exceptions (arrows).
- Fig. 11.** Varied appearance of trophoblast: large blastomeres, sometimes showing areas with few microvilli (white arrow), and small blastomeres (black arrow heads), near the zona (top) of a hatching blastocyst. Same treatment and age as in Fig. 9.
- Fig. 12.** Usual appearance of the microvilli on a hatching blastocyst cultured from Day 9 to Day 10. A few cytoplasmic blebs are visible; the zona edge is at lower left corner.

PLATE 1

(Facing p. 12)

PLATE 2