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# The behaviour of cow blastocyst *in vitro*: cinematographic and morphometric analysis

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## INTRODUCTION

The fact that the preimplantation mammalian embryo can hatch *in vitro* indicates that the forces involved originate within the embryo itself. In the mouse, Cole (1967) thought that the physical force generated by the alternating expansion and contraction of the blastocyst, over a period of many hours, was apparently responsible for tearing a hole in the zona pellucida through which the blastocyst ultimately escaped. Recently, Liu Bin & Mulnard (1980) have observed three types of behaviour for the mouse blastocyst: "discontinuous expansion interrupted by rapid contractions and followed, or not, by hatching; practically continuous expansion followed by hatching". These observations demonstrated that the pulsatile activity of the blastocyst was not a pre-requisite for hatching. In the cow, Massip & Mulnard (1980) have reported the third type of behaviour mentioned above, for normal embryos, but their experiment was limited to a very small number. In order to study more precisely the behaviour of the cow blastocyst during the hatching process *in vitro*, a time-lapse cinematographic investigation, completed by a morphometric analysis, was undertaken.

## MATERIALS AND METHODS

Embryos were recovered surgically (Rowson, Moor & Lawson, 1969) or non-surgically (Newcomb, Christie & Rowson, 1978) on day 7 (Day 0 = day of oestrus) from super-ovulated heifers of mixed breeds. Super-ovulation treatment and flushing medium were as indicated previously (Massip & Mulnard, 1980). After recovery, the embryos at the early blastocyst stage were washed in fresh flushing medium and cultured by the flat capillary tube technique designed for continuous cinematographic surveillance (Mulnard, 1967). The culture medium was a modified Krebs–Ringer bicarbonate supplemented with lactate, pyruvate and 20 % heat-inactivated sheep serum (Massip & Mulnard, 1980). The embryos were filmed for four to five days in the same medium but were left in culture until six or seven days. Negatives of the films were analysed on a moviola 16 mm which allows drawing and printing of the selected frames. Diameter, perimeter and surface of each drawing were measured with an automatic image analyser system (Leitz ASM).

## RESULTS

### *Rate, time and duration of hatching in vitro*

Of the 27 embryos cultured, 19 (70·4 %) hatched normally i.e. by shedding the cracked zona pellucida, two hatched partially by herniation through a reduced opening of the zona, one remained inert and five did not hatch.

Rupture of the zona occurred after about 12 hours of culture in two embryos, 23 hours in six, 36 hours in ten and 51 hours in one. The mean ± S.D. perimeter of the embryos, without the zona, at the beginning of the culture period was 433 ± 41·2 $\mu$m, At the time of rupture of the zona it was 708 ± 88 $\mu$m, an increase of 63 %.

Hatching was complete within 48 hours of culture in 15 embryos, 72 hours in three and 96 hours in one. This corresponds to days 9–10 after oestrus. The mean ± S.D. perimeter after hatching was 914 ± 75 $\mu$m (an increase of 29 % compared to the perimeter at the time of zona rupture). The hatching process took from 1 to 22 hours (12·05 hours ± 6·42 hours). The duration of the culture period before rupture of the zona did not influence the duration of hatching. After hatching, a proportion of embryos from a particular animal looked morphologically normal for a long time (until 6 days of culture) while others degenerated soon after hatching.

### *Modalities of blastocyst expansion and hatching.*

Of the 19 blastocysts that hatched normally, five (26·3 %) showed no pulsatile activity during expansion and hatching while the others underwent few contractions (no more than three) before rupture of the zona and/or during hatching (Table 1). During contractions, the blastocoelic cavity sometimes disappeared. Pulsation movements were still observable after the embryos had escaped from the zona. Figures 1 and 2 illustrate these different types of behaviour. Contractions of the blastocysts occupied about 17 minutes while the re-expansion took place approximately linearly, but much more slowly, occupying about 9–10 hours. The zona pellucida diminished in thickness as the blastocyst expanded and showed a slight elasticity, retracting rather slowly following the sudden contraction of the blastocysts. It broke at the embryonic pole in 4 blastocysts, at the abembryonic pole in one and at any point of the mural trophoblast in the remaining 14. In the 19 cases, the blastocysts escaped from the zona through a large slit, by active protrusion and without preliminary rotation. The duration of hatching was longer when pulsations occurred during hatching. The appearance of the zona after escape of the blastocyst, with the characteristic tear through which the blastocyst had passed, was very similar to that of empty zonae which could be flushed from cow uteri after day 9 of oestrus.

Two blastocysts hatched incompletely by herniation through a reduced opening

Table 1. *Number of embryos that have pulsed, or not, before and/or during hatching; and the number of pulsation movements*

| Pulsation movements before rupture of the zona | Pulsation movements during hatching | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | Total |
| 0 | 5 | 5 | 2 | 1 | 13 |
| 1 | 4 | 0 | 0 | 0 | 4 |
| 2 | 1 | 1 | 0 | 0 | 2 |
| Total | 10 | 6 | 2 | 1 | 19 |

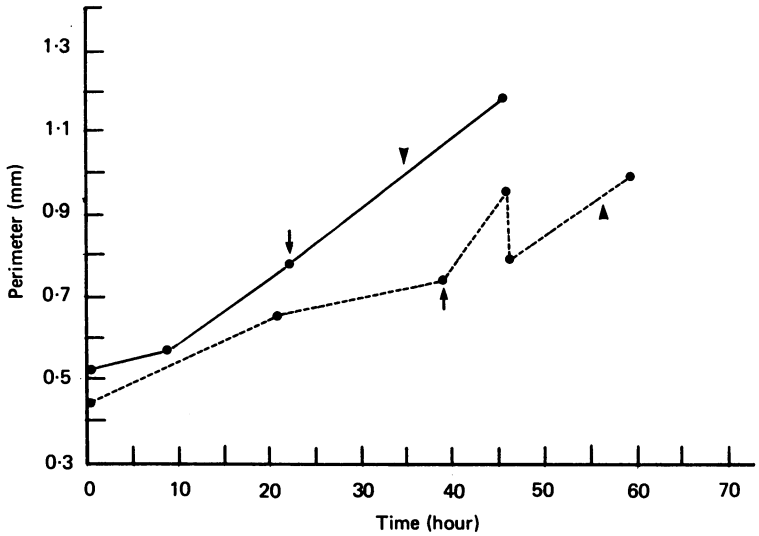


Fig. 1. Behaviour of two blastocysts hatching *in vitro*. Solid line: continuous expansion followed by rupture of the zona (arrow) and hatching (arrowhead). Dotted line: continuous expansion, but hatching is interrupted by a cycle of contraction and expansion.

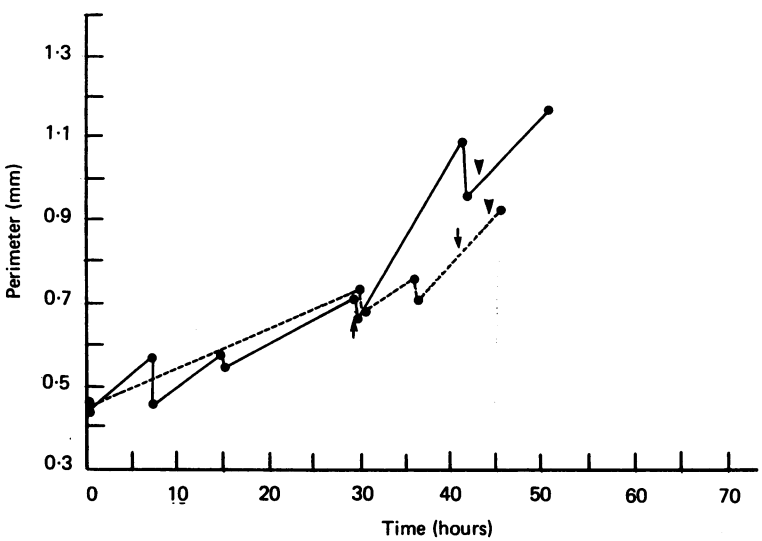


Fig. 2. Behaviour of two blastocysts hatching *in vitro*, one exhibiting a pulsatile activity during both expansion and hatching (solid line), the other during expansion only (dotted line).

of the zona. In these cases, hatching stopped when approximately half of the embryo had protruded outside, the blastocyst taking a diabolo shape. In one of them, the inner cell mass was divided in two equal parts by the herniation (Fig. 3). In the two blastocysts, the herniated part exhibited a rhythmic series of contractions and expansion.

Five embryos out of 27 (18·5 %) did not hatch. Their expansion was interrupted by several rapid contractions and re-expansions until they finally collapsed (Fig. 4). Contractions occupied about 13 minutes and re-expansion 6 hours.

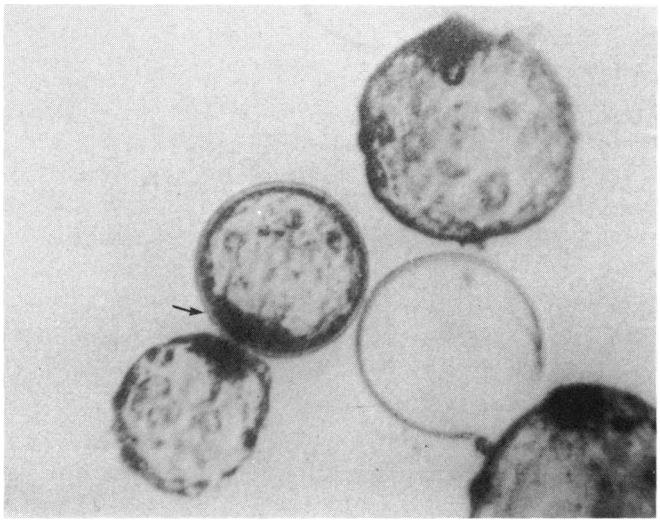

Fig. 3. Hatching of a blastocyst by herniation through a reduced opening of the zona (arrow). The inner cell mass is divided in two parts. × 105.

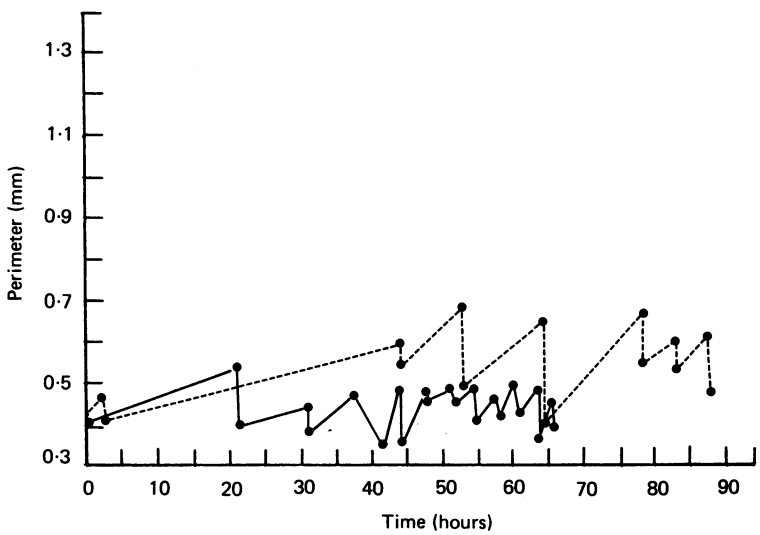


Fig. 4. Behaviour of two blastocysts which did not hatch *in vitro*. They underwent a rhythmic series of contractions and expansions.

The 27 embryos examined were obtained from five animals and it was interesting to note that the general behaviour of embryos from a particular animal was quite similar and often differred from those from another animal. For example, seven blastocysts out of eight, three out of four and two out of three recovered from three donor heifers exhibited pulsation movements followed by normal hatching while three out of four embryos collected from a fourth donor expanded and hatched without interruption. On the contrary, five out of seven embryos of the last donor animal showed a limited expansion and contracted several times before degeneration, while the two remaining embryos hatched normally.

## DISCUSSION

The rate of hatching *in vitro* for day 7 cow embryos in our culture conditions (70·4 %) is slightly lower than that reported by Ménézo (1976); Renard, Du Mesnil Du Buisson, Wintenberger-Torres & Ménézo (1976) and Renard & Heyman (1979) using Ménézo's medium (80·0 % and 74·0 %, respectively).

The time of hatching *in vitro* corresponds approximately with the time of zona loss *in vivo* (9–10 days after oestrus; Renard *et al.* 1976; Fléchon & Renard, 1978; Massip & Mulnard, 1980). Hatching itself is an active process, as demonstrated by Fléchon & Renard (1978) and Massip & Mulnard (1980).

The fact that embryos of a particular animal remain morphologically normal for varying periods in culture probably reflects differences in the viability of eggs for that animal.

Cow blastocysts and expanded blastocysts examined immediately after flushing from the uterus, or a few hours later (after having been left on the bench at room temperature) show a variety of forms. Some appear fully expanded, with the trophoblast flattened and closely adherent to the zona, while others are collapsed inside an intact zona or a split zona (Fléchon & Renard, 1978). Intermediates between these extremes are also found. This range of appearances parallels that undergone by individual blastocysts during the cycles of contraction and re-expansion described previously and suggests that similar cycles occur *in utero*. However some blastocysts can probably also become contracted within the zona as a result of physiological stress, e.g. temperature shock (Cole, 1967).

Previous workers have demonstrated the existence of a rhythmic series of contractions and expansions of the blastocysts of the rabbit (Lewis & Gregory, 1929), mouse (Kuhl & Friedrich-Freska, 1936; Cassini, 1962; Cole & Paul, 1965; Cole, 1967; Mulnard, 1967), rat (Bitton-Casimiri, Brun & Psychoyos, 1970) and guinea-pig (Blandau, 1971). Most of these workers consider this pulsatile activity to be a normal phenomenon leading to the rupture of the zona (Cole & Paul, 1965; Cole, 1967; Orsini & McLaren, 1967; Bitton-Casimiri *et al.* 1970).

Our observations demonstrate that, in the cow as in the mouse (Liu Bin & Mulnard, 1980), the pulsatile activity of the blastocyst is not a necessary condition either for rupture of the zona or for hatching because 13 blastocysts out of 19 (68·4 %, Table 1) had an uninterrupted expansion before zonal rupture, and 5 out of them did not pulse at all during hatching. It is clear, also, that a moderate pulsatile activity is not incompatible with normal hatching. On the other hand, when expansion is frequently interrupted by repeated contractions, hatching does not occur even when the zona is broken, as observed in occasional cases (Massip & Mulnard, 1980).

The extensive expansion of the blastocyst is dependent upon mechanisms for the accumulation and retention of fluid in the blastocoele cavity (Hastings & Enders, 1975). The accumulation of fluid depends upon the development of water transport mechanisms across the trophectoderm (Biggers, Borland & Powers, 1977) and it seems likely that prostaglandins of the E series, produced endogenously, are necessary for the formation of blastocoele fluid in the mouse because their antagonists can inhibit hatching (Biggers, Leonov, Baskar & Fried, 1978). Retention of fluid implicates the presence and integrity of a continuous junctional complex between trophoblast cells (Enders, 1971; Hastings & Enders, 1975; Ducibella, Albertini, Anderson & Biggers, 1975; Ducibella, 1977).

When hatching is preceded and/or accompanied by contractions and re-expansion this means the loss of a certain amount of water from the embryo, probably because the seal at the cell–cell junctions is incomplete in some blastocysts.

The abnormal expansion encountered in the blastocysts which fail to hatch could be attributed to a functional and structural immaturity or anomaly of the trophectoderm leading to impaired accumulation and retention of fluid in the blastocoelic cavity unless it were of metabolic origin. Renard, Philippon & Ménézo (1980) have found differences in the metabolic activity, particularly concerning glucose uptake, between the embryos. Of eleven out of fifty nine 10–11 day embryos (18·7 %) which failed to enlarge after a 20 hours culture period, two did and nine did not take up glucose from the medium. In the mouse, glucose is a necessary factor *in vitro* for the hatching of blastocysts (Wordinger & Brinster, 1976).

Whatever the causes of this anomaly of development, this study confirms that hatching *in vitro* is a good criterion for testing the developmental potentialities of cow embryos.

Herniation of the blastocyst through a reduced opening of the zona was reported in the guinea-pig (Blandau, 1971) and the mouse (Liu Bin & Mulnard, 1980). It is rarely followed by complete hatching. Usually, the process stops when half of the blastocyst is outside. When the inner cell mass is divided in two parts by this herniation (as illustrated in Figure 3), one may suppose that this process could lead to an undoubling of the embryo and thereby to the formation of biochorionic monozygotic twins, provided that the part of the embryo enclosed in the zona could get out. From our observations, we are unable to offer an explanation concerning this mechanism.

The differences in the behaviour of the embryos between donor animals shows that there is a large variation in the viability of eggs of the same stage, put in the same conditions but coming from different cows. This observation confirms those of Trounson, Willadsen & Rowson (1976), Seidel (1977) and Renard & Heyman (1979).

## SUMMARY

The behaviour of the cow blastocyst *in vitro* was studied by time-lapse cinematography and analysed by morphometry. Three types of behaviour were observed: continuous expansion followed by hatching; discontinuous expansion interrupted by few contractions and followed by hatching; discontinuous expansion interrupted by several rapid contractions without hatching. This demonstrated that the pulsatile activity of the blastocyst is not a necessary condition of hatching but also that only a moderate pulsatile activity is compatible with normal hatching. The time of hatching *in vitro* corresponded approximately with the time of zona loss *in vivo* (9–10 days). Rupture of the zona occurred at any point of the trophoblast layer. Hatching by herniation through a reduced opening of the zona was occasionally observed. The behaviour of the embryos from a particular animal was very similar but differences were noted between embryos from different animals.

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## REFERENCES


BIGGERS, J. D., BORLAND, R. M. & POWERS, R. D. (1977). Transport mechanisms in the preimplantation mammalian embryo. In *The Freezing of Mammalian Embryos*, Ciba Foundation Symposium, pp. 129–146. Amsterdam: Elsevier/Excerpta Medica/North Holland.

BIGGERS, J. D., LEONOV, B. V., BASKAR, J. F. & FRIED, J. (1978). Inhibition of hatching of mouse blastocysts *in vitro* by prostaglandin antagonists. *Biology of Reproduction* **19**, 519–533.

BITTON-CASIMIRI, V., BRUN, J. L. & PSYCHOYOS, A. (1970). Comportement *in vitro* des blastocystes du 5ème jour de la gestation chez la rate; étude microcinématographique. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **270**, 2979–2982.

BLANDAU, R. (1971). Culture of guinea pig blastocyst. In *The Biology of the Blastocyst* (ed. R. J. Blandau), pp. 59–70. Chicago: University of Chicago Press.

CASSINI, A. (1962). Osservazioni microcinematografische sulla segmentatione dell'uovo di topo. *Monitore zoologico italiano (suppl.)* **70**, 208–214.

COLE, R. (1967). Cinematographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *Journal of Embryology and Experimental Morphology* **17**, 481–490.

COLE, R. & PAUL, J. (1965). Properties of cultured preimplantation mouse and rabbit embryos and cell strains derived from them. In *Preimplantation Stages of Pregnancy* (ed. G. E. W. Wolstenholme), Ciba Foundation Symposium, pp. 82–112. London: Churchill.

DUCIBELLA, T. (1977). Surface changes of the developing trophoblast cell. In *Development in Mammals* (ed. M. H. Johnson) pp. 5–31. Amsterdam, New York, Oxford: North-Holland Publishing Co.

DUCIBELLA, T., ALBERTINI, D. F., ANDERSON, E. & BIGGERS, J. D. (1975). The preimplantation mammalian embryo: characterization of intercellular junctions and their appearance during development. *Developmental Biology* **45**, 231–250.

ENDERS, A. C. (1971). The fine structure of the blastocyst. In *Biology of the Blastocyst* (ed. R. J. Blandau), pp. 71–94. Chicago: University of Chicago Press.

FLÉCHON, J. E. & RENARD, J. P. (1978). A scanning electron microscope study of the hatching of bovine blastocysts *in vitro*. *Journal of Reproduction and Fertility* **53**, 9–12.

HASTINGS, R. A. & ENDERS, A. C. (1975). Junctional complexes in the preimplantation rabbit embryo. *Anatomical Record* **181**, 17–34.

KUHL, W. & FRIEDRICH-FRESKA, H. (1936). Richtungskörperbildung und Furchung des Eies sowie das Verhalten des Trophoblasten der weissen Maus. *Zoologischer Anzeiger (suppl.)* **9**, 187–195.

LEWIS, W. H. & GREGORY, P. W. (1929). Cinematographs of living developing rabbit eggs. *Science, N.Y.* **69**, 226–227.

LIU BIN & MULNARD, J. (1980). Analyse cinématographique et morphométrique du comportement *in vitro* du blastocyste de la souris. *Archives de Biologie* **91**, 37–48.

MASSIP, A. & MULNARD, J. (1980). Time-lapse cinematographic analysis of hatching of normal and frozen-thawed cow blastocysts. *Journal of Reproduction and Fertility* **58**, 475–478.

MÉNÉZO, Y. (1976). Milieu synthétique pour la survie et la maturation des gamétes et pour la culture de l'œuf fécondé. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **282**, 1967–1970.

MULNARD, J. (1967). Analyse microcinématographique du développement de l'œuf de souris du stade II au blastocyste. *Archives de Biologie* **78**, 107–138.

NEWCOMB, R., CHRISTIE, W. B. & ROWSON, L. E. A. (1978). Non surgical recovery of bovine embryos. *Veterinary Record* **102**, 414–417.

ORSINI, W. M. & MCLAREN, A. (1967). Loss of the zona pellucida in mice, and the effect of tubal ligation and ovariectomy. *Journal of Reproduction and Fertility* **13**, 485–499.

RENARD, J. P., DU MESNIL DU BUISSON, F., WINTENBERGER-TORRES, S. & MÉNÉZO, Y. (1976). *In vitro* culture of cow embryos from day 6 to day 7. In *Egg Transfer in Cattle* (ed. L. E. A. Roswon), pp. 159–164. Luxembourg: Commission of the European Communities.

RENARD, J. P. & HEYMAN, Y. (1979). Variable development of superovulated bovine embryos between day 6 and day 12. *Annales de biologie animale, biochimie et biophysique* **19** (5), 1589–1598.

RENARD, J. P., PHILIPPON, A. & MÉNÉZO, Y. (1980). *In-vitro* uptake of glucose by bovine blastocysts. *Journal of Reproduction and Fertility* **58**, 161–164.

ROWSON, L. E. A., MOOR, R. M. & LAWSON, R. A. S. (1969). Fertility following egg transfer in the cow; effect of method, medium and synchronization of oestrus. *Journal of Reproduction and Fertility* **18**, 517–523.

SEIDEL, G. E. (1977). Short term maintenance and culture of embryos. In *Embryo Transfer in Farm Animals* (ed. K. J. Betteridge), pp. 20–24. Canada Department of Agriculture, Monograph 16.

TROUNSON, A. O., WILLADSEN, S. M. & ROWSON, L. E. A. (1976). The influence of *in vitro* culture and cooling on the survival and development of cow embryos. *Journal of Reproduction and Fertility* **47**, 367–370.

WORDINGER, R. J. & BRINSTER, R. L. (1976). Influence of reduced glucose levels on the *in vitro* hatching, attachment and trophoblast outgrowth of the mouse blastocyst. *Developmental Biology* **53**, 294–296.