Time-lapse cinematographic analysis of hatching of normal and frozen–thawed cow blastocysts*

A. Massip and J. Mulnard†

Department of Obstetrics and Animal Reproduction, Faculty of Veterinary Medicine, 45 rue des Vétérinaires, 1070 Brussels, and †Free University of Brussels Medical School, Department of Anatomy and Embryology, 97 rue aux Laines, 1000 Brussels, Belgium

Summary. This paper describes time-lapse cinematography on cattle blastocysts hatching from the zona *in vitro*. Four normal morulae were taken on Day 6 and cultured for 5 days. During this period 3 formed normal blastocysts of which 2 hatched from the zona without undergoing any contractions. Early blastocysts were taken on Day 7, frozen and stored at low temperature. After thawing they were cultured; only 1 of the 11 behaved like the normal embryos, and the others underwent contractions and failed to hatch. These contractions could be interpreted as a sign of an alteration of the trophoblastic structure and properties.

Introduction

Renard, du Mesnil du Buisson, Wintenberger-Torrès & Ménéz (1976) have used the ability to hatch *in vitro* as a criterion for testing the short-term developmental potentialities of cow blastocysts and the adequacy of the culture conditions.

In the present study, cow blastocyst formation and hatching *in vitro* were analysed by continuous time-lapse cinematography. The behaviour of embryos frozen–thawed at the early blastocyst stage was then studied by the same technique to assess their ability to resume blastocoele expansion and hatch *in vitro*.

Materials and Methods

One heifer and 3 culled cows of mixed breeds were used to provide fertilized eggs. Superovulation was induced by a single intramuscular (i.m.) injection of 2200 i.u. PMSG (Folligon: Intervet) for the heifer and 3000 i.u. PMSG for the cows between Days 9 and 12 of the cycle (Day 0 = day of oestrus) followed 48 h later by an i.m. injection of 1000 µg Cloprostenol (I.C.I. 80,996), a prostaglandin analogue. The animals were detected in oestrus with a bull 48 h after the last injection and were inseminated 3 times at 12 h intervals with frozen semen in 0.25 ml French straws, each containing $\pm 2 \times 10^7$ spermatozoa. One straw was used for the first insemination, 2 for the second and 1 for the last. They were mounted in a Cassou Insemination gun. The cows were slaughtered on Day 6 (N = 1) or 7 (N = 2) of the induced cycle. The uteri were recovered about 25 min after the animals were killed, placed in a clean towel and transported to the laboratory in a closed plastic container without particular precautions. The uterine horns were trimmed and perfused with 30–50 ml phosphate-buffered saline (PBS) (Trounson,

* Copies of the film are obtainable from Dr A. Massip.

Willadsen & Rowson, 1976). The interval from slaughter to flushing varied from 40 to 60 min. Eggs were obtained from the heifer on Day 7 using the surgical method of Rowson, Moor & Lawson (1969) and PBS as the flushing medium. The morulae recovered on Day 6 were used to investigate blastocoele formation and hatching of normal embryos *in vitro*. The embryos were transferred to a small rubber-stoppered glass test-tube (1 × 7.5 cm) containing 1–2 ml PBS supplemented with 20% heat-inactivated sheep serum and stored for 2–3 h at ambient temperature (18–22°C) before being placed in the culture medium. This was a modified Krebs–Ringer solution supplemented with 20% heat-inactivated sheep serum. The composition (per litre) was: 6.85 g NaCl, 0.35 g KCl, 0.29 g MgSO₄ · 7H₂O, 0.16 g NaH₂PO₄ · H₂O, 0.71 g sodium lactate, 0.83 g glucose, 30 mg sodium pyruvate, 2 g NaHCO₃, 20 mg phenol red, 0.8 mg penicillin and 0.5 mg streptomycin. The pH was adjusted and maintained at 7.4 by a mixture of 90% N₂, 5% O₂ and 5% CO₂. The medium was not changed during the course of the experiments. The apparatus and the flat capillary tube culture technique which permits continuous cinematographic surveillance were as previously described (Mulnard, 1967). The film record was taken at a frame interval of 80 sec during 5 days. To study the resumption of development and hatching of frozen–thawed embryos, 11 early blastocysts obtained on Day 7 by surgery (N = 4) from the heifer or at slaughter (N = 7) from the cows were immediately frozen in 0.25 ml French straws by the “two step” freezing method of Willadsen, Polge & Rowson (1978). The freezing medium was 1.5 M-DMSO in PBS. The embryos were thawed rapidly after a few hours to 14 days at liquid nitrogen temperature by agitating the straws in water at ±25°C. Immediately after thawing the embryos were transferred to freshly prepared 1.5 M-DMSO in PBS. DMSO was

PLATE 1

Sequences extracted from a time-lapse cinematographic record of the development of a normal cow embryo from morula to complete blastocyst hatching *in vitro*. The times after explantation are indicated in parentheses.

Figs 1–6. Blastocoele formation and expansion (3, 11, 27, 35, 54 and 60 h).

Fig. 7. Rupture of the zona pellucida (arrow) (62 h).

Fig. 8. Start of hatching (64 h).

Figs 9–13. Escape of the blastocyst from the ruptured zona (67, 74, 74½, 74½ and 77 h).

Fig. 14. The blastocyst after hatching (78 h).

PLATE 2

Behaviour *in vitro* of cow embryos frozen–thawed at the early blastocyst stage (7 days).

Figs 15–23. Sequences extracted from a time-lapse cine-record (0, 19, 33, 42, 42½, 52, 54, 59 and 60 h after explantation), showing re-expansion interrupted by repeated contractions. The zona pellucida was ruptured (Fig. 18) but the blastocyst failed to hatch.

Fig. 15. Contracted blastocyst at the start of the culture.

Figs 16–17. Expansion of the blastocoele.

Fig. 18. Rupture of the zona pellucida.

Fig. 19. First contraction.

Fig. 20. Re-expansion.

Fig. 21. Second contraction.

Fig. 22. Second re-expansion.

Fig. 23. Third contraction.

Figs 24–29. Time-lapse cine-record (18, 53, 60, 60½, 61 and 69 h after explantation), showing uninterrupted re-expansion and normal hatching of a frozen–thawed early blastocyst.

PLATE 1

(Facing p. 476)

PLATE 2

removed from the embryos in a step-wise fashion at room temperature (1.5 M:5 min, 1.25 M, 1.0 M, 0.75 M, 0.5 M, 0.25 M:10 min each, PBS). After 30 min to 1 h in pure PBS the embryos were placed in culture and filmed for 5 days in the same conditions as for normal embryos.

Results

Formation and hatching of normal blastocysts (Plate 1)

Of the 4 compacted morulae explanted at Day 6, 2 were at the early blastocyst stage on the 2nd day of culture, 1 hatched on the 3rd day and the other on the 4th day. The blastocoele was formed by fusion of small cavities which appeared eccentrically (Pl. 1, Figs 1 and 2). The blastocyst then expanded continuously and regularly, distending the zona pellucida which became gradually thinner (Pl. 1, Figs 3–6). After 62 h of culture, the zona broke rapidly and the blastocyst became slightly elongated (Pl. 1, Fig. 7). Hatching began immediately by protrusion of the blastocyst through the slit of the zona. Small cytoplasmic protrusions through a small gap in the zona were occasionally observed immediately before or at the time of zona rupture (Pl. 1, Fig. 7: arrow). Except during the very first stages of its formation (Pl. 1, Fig. 2) the blastocyst exhibited no contraction, the expansion being uninterrupted until and after hatching (Pl. 1, Fig. 14).

The other two morulae underwent blastocoele formation and expansion but failed to hatch. They exhibited moderate movements of contraction and re-expansion before their development stopped.

Resumption of development of frozen-thawed early blastocysts (Plate 2)

After thawing, the development of these 11 blastocysts was as follows: 5 remained collapsed or showed only an abortive tendency to expand; 4 expanded normally but exhibited repeated movements of contraction and re-expansion until final collapse, the zona remaining intact; 1 blastocyst succeeded in breaking the zona pellucida but failed to hatch. Cinematographic analysis of this last blastocyst showed a succession of several rapid contractions followed by slow re-expansion before and mainly after rupture of the zona (Pl. 2, Figs 15–23). The remaining blastocyst (Pl. 2, Figs 24–29) showed normal behaviour in that it expanded regularly and distended the zona, thus hatching by a process identical to that of a normal, unfrozen embryo. No contractions were observed. The chronology of expansion and hatching was the same as for an unfrozen blastocyst.

Discussion

Time-lapse cinematographic analysis of normal blastocyst development in the cow shows that the blastocoele is formed by a mechanism which does not differ essentially from that described for other mammalian embryos, e.g. the mouse (Mulnard, 1967): small cavities appear eccentrically and rapidly coalesce between the trophectoderm and inner cell mass. The time of hatching of normal cow blastocysts *in vitro* corresponded approximately with the time of zona loss *in vivo* (9–10 days after oestrus: Renard *et al.*, 1976).

Hatching of a preimplantation mammalian embryo by the escape of the blastocyst through a localized rupture in the zona pellucida was first observed *in vitro* by Lewis & Gregory (1929) in their pioneer cinematographic study of the preimplantation development of the rabbit and was confirmed in the same species by Pincus & Werthessen (1938), Adams (1970) and Kane (1973). Hatching of the mouse blastocyst *in vitro* is also extensively documented (Lewis & Wright, 1935; Hammond, 1949; Whitten, 1957; Mintz, 1962; Brinster, 1963; Cole & Paul, 1965; Cole, 1967). However, only one study of the hatching of cow blastocysts *in vitro* has been reported (Fléchon & Renard, 1978).

The present study confirms the observations of Fléchon & Renard (1978) that the zona pellucida does not show signs of uniform lysis but is perforated in one place only. Normal hatching of the cow embryo is operated by active escape of the blastocyst through a slit which is formed by a rapid rupture of the zona pellucida. It is not preceded, or followed, by repeated contractions and re-expansions as have been described for the mouse (Cole, 1967). Each time such pulsation movements were present in the cow blastocysts, the hatching process did not occur even if the zona was ruptured. The repeated pulsation movements which were observed in most of the frozen-thawed embryos and, to a lesser degree, in some of the normal blastocysts which did not hatch could be the result of alterations of the trophoctoderm and in particular of the junctional complexes which normally develop between the trophoblastic cells. In the absence of systematic controls (due to scarcity of material), our observations do not permit any conclusion about the factors responsible for such alterations. It can only be postulated that if freezing and thawing do not necessarily alter the structure of the blastocyst, since normal hatching is possible, it might aggravate pre-existent alterations possibly caused by manipulations of the embryos. On the other hand, it is clearly necessary to transplant blastocysts which have exhibited abnormal behaviour and failed to hatch *in vitro* to know whether they are capable of normal development *in vivo*. Meanwhile we agree with Renard *et al.* (1976) that the capacity to hatch *in vitro* provides a better criterion for assessing the integrity of 7-day blastocysts than expansion only. Willadsen *et al.* (1978) found that the majority of surviving cow embryos after freezing and thawing contained a proportion of degenerate cells when examined after fixation and staining by the end of the culture period. This could also explain the different behaviour patterns observed by cinematography in the present study.

We gratefully acknowledge the technical assistance of W. Zwijsen and the financial support of I.R.S.I.A.

References

- Adams, C.E. (1970) The development of rabbit eggs after culture *in vitro* for 1–4 days. *J. Embryol. exp. Morph.* **23**, 21–34.
- Brinster, R.L. (1963) A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Expl Cell Res.* **32**, 205–208.
- Cole, R.J. (1967) Cinematographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J. Embryol. exp. Morph.* **17**, 481–490.
- Cole, R.J. & Paul, J. (1965) Properties of cultured pre-implantation mouse and rabbit embryos and cell strains derived from them. In *Preimplantation Stages of Pregnancy*, pp. 82–112. Eds G. Wolstenholme & M. O'Connor. J. & A. Churchill, London.
- Fléchon, J. E. & Renard, J.P. (1978) A scanning electron microscope study of the hatching of bovine blastocysts *in vitro*. *J. Reprod. Fert.* **53**, 9–12.
- Hammond, J., Jr (1949) Recovery and culture of tubal mouse ova. *Nature, Lond.* **163**, 28–29.
- Kane, M.T. (1973) Influence of the mucin coat on rabbit blastocysts in culture. *J. Physiol., Lond.* **236**, 30–31.
- Lewis, W.H. & Gregory, P.W. (1929) Cinematographs of living developing rabbit eggs. *Science, N.Y.* **69**, 226–229.
- Lewis, W.H. & Wright, E.S. (1935) On the early development of the mouse egg. *Contr. Embryol. Carneg. Inst.* **25**, 115–144.
- Mintz, B. (1962) Experimental study of the developing mammalian egg: removal of the zona pellucida. *Science, N.Y.* **138**, 594–595.
- Mulnard, J.G. (1967) Analyse microcinématographique du développement de l'oeuf de souris du stade II au blastocyte. *Archs Biol., Liège* **78**, 107–138.
- Pincus, G. & Werthessen, C.H. (1938) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. *J. exp. Zool.* **78**, 1–18.
- Renard, J.P., du Mesnil du Buisson, F., Wintenberger-Torrès, S. & Ménéz, Y. (1976) *In vitro* culture of cow embryos from Day 6 to Day 7. In *Egg Transfer in Cattle*, pp. 159–164. Ed. L. E. A. Rowson. Commission of the European Communities, Luxembourg.
- 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. *J. Reprod. Fert.* **18**, 517–523.
- 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. *J. Reprod. Fert.* **47**, 367–370.
- Whitten, W.K. (1957) The effect of progesterone on the development of mouse eggs *in vitro*. *J. Endocr.* **16**, 80–85.
- Willadsen, S.M., Polge, C. & Rowson, L.E.A. (1978) *In vitro* storage of cattle embryos. In *Control of Reproduction in the Cow*, pp. 427–436. Ed. J. M. Sreenan. Commission of the European Communities, Luxembourg.

Received 13 September 1979