

Original article

Two culture systems showing a biphasic effect on ovine embryo development from the 1–2 cell stage to hatched blastocysts

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(Received 31 October 1994; accepted 18 September 1995)

Summary — This study compared the effect of using either CZB or TCM 199 media on both the development of 1–2 cell ovine embryos from superovulated ewes to the blastocyst stage (*Experiment 1*), and the hatching process of ovine blastocysts developed *in vitro* (*Experiment 2*). For the first 5 d, the CZB medium showed higher rates of embryo development than the TCM 199 medium ($p < 0.001$). The embryos reaching the > 16 cell stage were 79 vs 52% and 74 vs 20% with or without an oviducal monolayer, respectively, and those reaching the blastocyst stage were 71 vs 46% and 46 vs 13% with or without cells. The CZB medium was less able to support the hatching process of the blastocysts obtained in the first experiment than was the TCM-199 medium + 10% FCS (fetal calf serum) with cells (31 vs 92%; $p < 0.001$) or without cells (13 vs 66%; $p < 0.001$). No blastocysts completely escaped from the zona pellucida (ZP) in the CZB medium compared with 80 and 61% in the TCM 199 medium with or without cells, respectively. In *Experiment 3*, 47% of the blastocysts migrated through the artificial opening of the ZP and hatched completely. After 24 h of culture in the CZB medium, however, they showed blastocoelic cavity breakdown. During the preliminary cleavages, the ovine embryos developed better in CZB medium than in TCM 199, but the latter was more efficient in promoting the hatching process of the blastocysts.

culture media / ovine embryo development / hatching

Résumé — Deux systèmes de culture montrant un effet biphasique dans le développement des embryons ovins du stade 1–2 cellules jusqu'à l'éclosion. Les effets d'un milieu de culture, CZB, et d'un milieu de culture, TCM 199 + 10% de sérum de veau fœtal, ont été observés sur le développement *in vitro* d'embryons d'ovins du stade 1–2 cellules au stade blastocyste (expérience 1) et dans le processus d'éclosion des blastocystes produits lors de l'expérience précédente (expérience 2). Le milieu CZB exerce un effet positif sur le développement embryonnaire par rapport au milieu TCM 199 après culture jusqu'au stade > 16 cellules (79 vs 52% et 74 vs 20%) ou jusqu'au stade blastocyste (71 vs 46% et 46 vs 13%) en présence de cellules et en l'absence de cellules tubaires respectivement. Le processus d'éclosion est plus faible dans le milieu CZB que dans le milieu TCM 199 soit en présence (31 vs 92%) soit en l'absence (13 vs 66%) de cellules tubaires respectivement. Dans le CZB (expérience

3), 47% des blastocystes éclosent par une fente artificielle dans la zone pellucide, mais, après 24 h de culture, la cavité blastocœlique se dégonfle. Ces résultats montrent que le milieu CZB est plus indiqué pour le développement précoce d'embryons ovins alors que le milieu TCM 199 est plus efficace au moment de la phase d'éclosion.

milieu de culture / développement embryonnaire / ovins / éclosion

INTRODUCTION

In mammals, most of the information on the morphological and biochemical changes of early embryonic division, including genomic activation, blastomere compaction, trophoblastic and inner cell mass differentiation, blastocoelic expansion and hatching from the zona pellucida (ZP), has been obtained from *in vitro* experiments. Two crucial events in the process of embryo development *in vitro* are the cleavage block and the escape of the blastocyst from the ZP.

Embryos of many species encounter an *in vitro* development block at different cleavage stages. These correspond to the autonomous transcription of the embryonic genome when culture conditions are sub-optimal (Bavister, 1988). In sheep, this block occurs during the 8- to 16-cell stage (Gandolfi and Moor, 1987) and may be partially overcome by co-culture in TCM-199 medium with oviductal-epithelial cells (Gandolfi and Moor, 1987; Rexroad and Powell, 1988), or in a synthetic oviduct fluid medium supplemented with 20% heat-inactivated human serum and 5% O₂ (Walker *et al*, 1988; Walker *et al*, 1992), or in CZB medium with or without oviductal monolayer (Ledda *et al*, 1991; McGinnis and Young, 1992).

Very few reports exist in the literature dealing with the process of ovine blastocyst hatching. Plasminogen activation to plasmin by the blastocyst may be a mechanism utilized by the embryo to produce a weakening or sublysis of the ZP. This facilitates hatching as it is concomitant with blastocoelic expansion (Menino and Williams, 1987; Menino *et al*, 1989). The process of

hatching may also be independent of protease action, and be due to the mechanical rupture of the ZP by cell proliferation (Kane, 1983).

In the present study, we examined the effect of different culture systems on the development of ovine embryos from the 1–2 cell to blastocyst stage and on their subsequent complete escape from the ZP. The different media used were CZB medium and TCM-199 supplemented with fetal calf serum (FCS) with or without oviductal-epithelial cells.

MATERIALS AND METHODS

Superovulation and embryo collection

Sarda ewes were superovulated by administering 16 mg of follicle-stimulating hormone (FSH-p, Sigma) every 12 h in 4 doses (6, 5, 3 and 2 mg) starting 24 h before removal of the intravaginal sponges (40 mg x 14 g of fluorogestone acetate (FGA), Intervet). Surgical collection of the embryos was done 48–52 h after the beginning of oestrus. The sheep were anaesthetized with thiopentale sodium and a midline ventral incision was made to exteriorize and flush the oviducts. The average number of corpora lutea from the 55 ewes treated and the recovery rate per ewe were 9.8 ± 4.6 and 72%, respectively. The number of embryos collected at the 1–2 cell stages was 325. No attempt was made to differentiate unfertilized oocytes from zygotes at this time.

The collection medium was Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma) and an antibiotic solution of 50 µg/ml streptomycin and 50 iu/ml penicillin (Sigma). All procedures were carried out at room temperature and the interval between flushing and culture was 1–2 h.

Establishment of oviductal cell monolayers

Ovine oviductal epithelial cells were collected from the oviducts of sheep slaughtered in the local abattoir.

The somatic cells were washed twice in PBS with BSA and an antibiotic solution of streptomycin-penicillin (50 µ/ml, 50 iu/ml) and were placed in TCM-199 with Earle's salts (Sigma) + 10% FCS (Gibco). The epithelial cells were cultured in 35 mm tissue culture dishes (Falcon) at 39°C in a humidified atmosphere (5% CO₂ in air).

The epithelial cell monolayers were used for co-culture when they had about 80% confluency, 3–4 d after incubation. Before being used for co-culture, the monolayers were washed 3 times with CZB medium to remove serum and TCM-199 with Earle's salt.

It was possible to maintain the viability of oviductal cells under these conditions although 5–10% of the cells did not attach.

Media and culture procedures

Ovine embryos at the 1–2 cell stage were cultured in 2 different media: TCM-199 medium, with Earle's salt supplemented with 10% FCS, 1 mM of glutamine (Bavister, 1987) and antibiotics, and CZB medium, containing 5 mg/ml BSA (Sigma, Fraction V). The CZB medium was prepared according to the standard method (Chatot *et al*, 1989) using cell culture reagents (Sigma) and ultrapure water (Milli Q system, Millipore). The medium was sterilized by passing it through a 0.22 µm Millix GV filter (Millipore). Glutamine was added immediately before use. A separate batch of CZB medium, containing 3.3 mM of glucose, was prepared and replaced 48 h after the beginning of the culture period. The osmolality of the CZB medium was adjusted to 280–285 mOs/kg with NaCl before use. The pH was 7.5 after equilibration in an atmosphere of 5% CO₂ in air.

Experiment 1

After being removed from the flushing medium, embryos at the 1–2 cell stage were randomly allo-

cated to the following treatment groups: (1) TCM-199 + 10% FCS over an epithelial cell monolayer; (2) TCM-199 + 10% of FCS; (3) CZB over an epithelial cell monolayer; and (4) CZB alone. The embryos were cultured in 35 mm tissue culture dishes (10–15 embryos per dish) with 2 ml medium. These were placed in an incubator at 39°C containing 5% CO₂ in air. The embryos were assessed daily for cleavage using an inverted microscope (Leitz Labovert) and the eggs that did not cleave after the first 24 h were fixed (acetic acid/alcohol 1:3) and stained (Iacmoid 1%) to verify whether or not fertilization had occurred. Those eggs that were unfertilized were excluded from the results. Morphological development was evaluated up to 5 d of culture when the embryos reached the blastocyst stage.

Experiment 2

This experiment was designed to evaluate the potentiality of the embryos developed in *Experiment 1* to hatch *in vitro*. For this purpose, unselected blastocysts developed in one of the different culture systems were monitored separately, after having been transferred into either TCM-199 + 10% FCS or CZB medium with or without oviductal-epithelial cells. Since only a few embryos developed in TCM 199 alone, the blastocysts obtained with this culture system were equally divided between CZB medium and TCM-199 + 10% FCS in the presence of oviductal cells. All the blastocyst groups were maintained up to 5 d in the same environment as in the first experiment and were checked every 12 h.

Experiment 3

Compacted morulae, developed in CZB and TCM-199 media, with somatic cells, as described in *Experiment 1*, were micromanipulated (Leitz) in order to form an incision of 180° on the ZP. They were subsequently cultured in CZB with an oviductal monolayer. For a period of up to 5 d all the blastocysts were checked every 6 h to see if they had escaped from the ZP. All the micromanipulation procedures were performed under an inverted microscope (Leitz) fitted with a micromanipulator (Leitz). The holding pipettes and microneedles were made from glass capillary tubing using a pipette puller (Clark Instruments).

Statistical analysis

Chi-square analysis was used to compare the effect of the different culture systems on the development and hatching process.

RESULTS

In *Experiment 1*, the CZB medium, supplemented with glucose 48 h after the beginning of culture, enabled a higher proportion ($p < 0.001$) of ovine embryos to develop to the > 16 cell stage than did the TCM-199 medium either with oviductal cells (79 vs 52%) or without (74 vs 20%). The greater efficacy of the simple medium was demonstrated by the larger ($p < 0.001$) number of blastocysts with oviductal cells (71 vs 46%) or without (46 vs 13%) (table I).

In *Experiment 2*, the continued maintenance in the CZB medium permitted a significantly lower number ($p < 0.001$) of blastocysts to hatch *in vitro* than those that were transferred into TCM-199 medium either with oviductal cells (31 vs 92%) or without (13 vs 66%). In the CZB medium culture systems, no blastocysts completed the hatching process and they demonstrated blastocoelic breakdown (figs 1 and 2). In the TCM-199 medium, however, either with the addition of 10% FCS alone or with cells,

a high proportion of the blastocysts hatched (61 and 80%). The somatic cells had a positive effect on the blastocyst hatching process ($p < 0.05$) when the TCM-199 medium was used (table II).

In *Experiment 3*, 81% of the manipulated morulae developed beyond the early cavitation stage and 65% migrated through the artificial opening. There were 47% that hatched completely. None of these blastocysts appeared to exhibit the phenomenon where the ZP thins which is usually found prior to the moment where the embryo begins its protrusion through the ZP. However, after 24–36 h of culture in the simple medium with somatic cells, blastocoelic cavity breakdown was observed (figs 3 and 4).

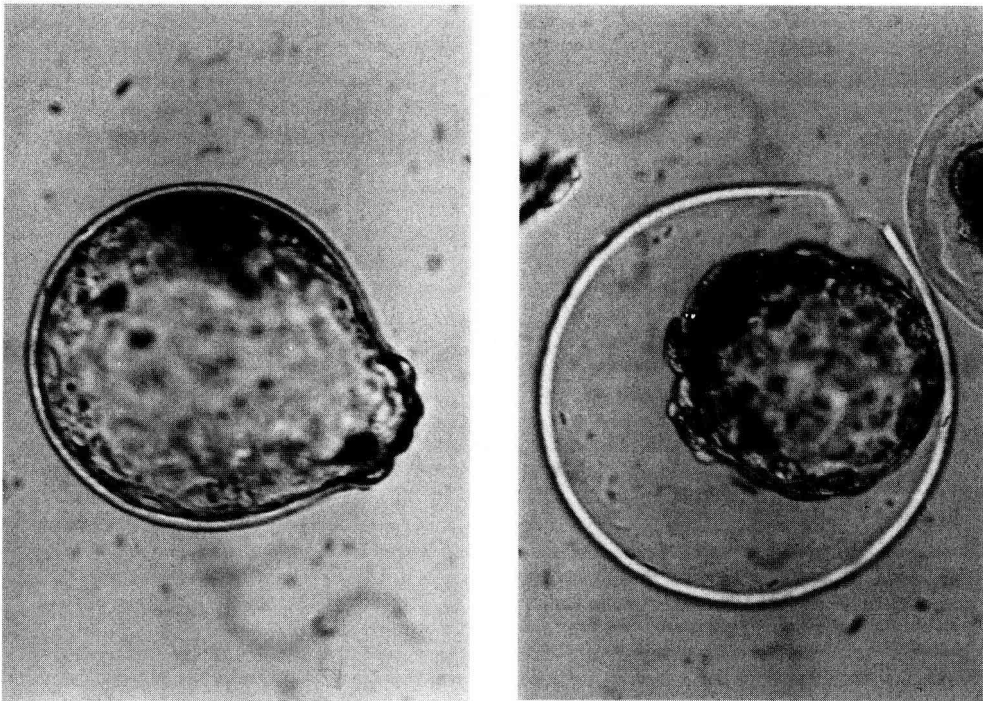
DISCUSSION

This study suggested that the CZB medium was superior to TCM-199 + 10% FCS in improving the cleavage from the 1–2 cell stage in ovine embryos to blastocyst formation. This is in agreement with observations from the first 48 h of bovine zygote culture (Hernandez *et al*, 1993). The CZB medium, however, is unable to promote the complete hatching process as successfully as the TCM-199 medium. The positive effect of CZB may have been the result of a vari-

Table I. Effect of different culture systems on development of ovine embryos from the 1–2 cell stage to the blastocyst.

Culture media	Somatic cells	No of embryos	> 16 cells (%)	Blastocysts (%)
CZB	–	85	63 (74.1) ^c	39 (45.9) ^a
	+	82	65 (79.3) ^a	58 (70.7) ^c
TCM 199 + 10% FCS	–	45	9 (20.0) ^d	6 (13.3) ^d
	+	113	59 (52.2) ^b	52 (46.0) ^b

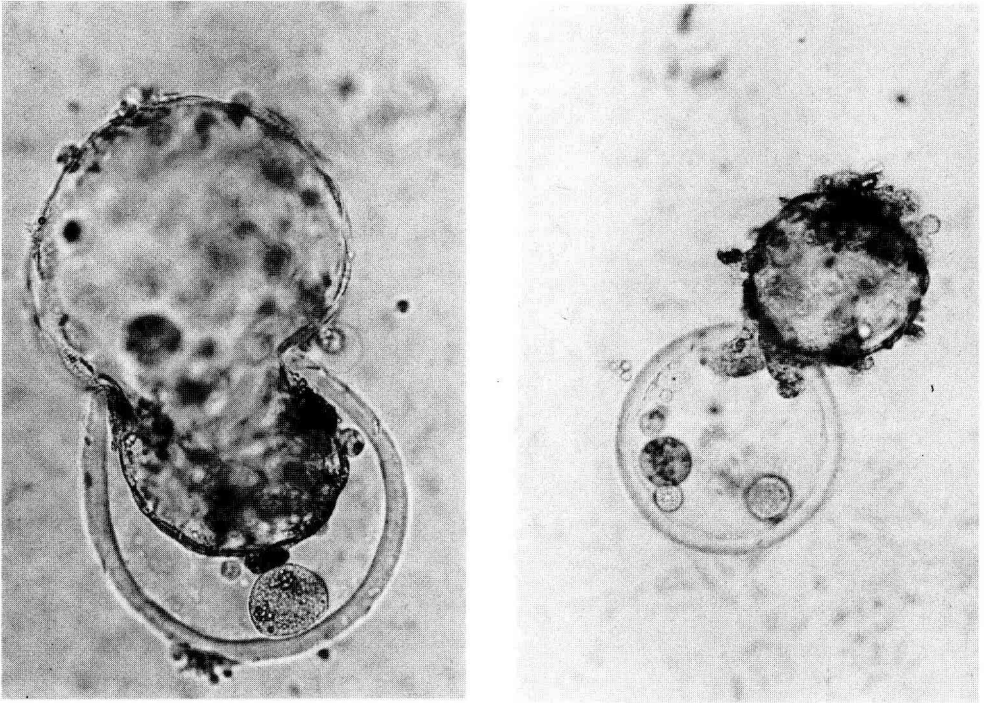
Within columns: a versus b and c versus d, $p < 0.001$.



Figs 1 and 2. 1. Hatching process of ovine blastocyst after 7 d of culture in CZB medium. 2. Blastocyst cultured in CZB medium for other 2 d underwent blastocoelic breakdown. Optical light magnification 250 x.

ation in the exposure time of the embryos to glucose. Glucose has an inhibitory effect on the development of ovine embryos if it is present during the early stages of culture (Ledda *et al*, 1991). As has been demonstrated in mice (Chatot *et al*, 1989), hamsters (Schini and Bavister, 1988) and cattle (Ellington *et al*, 1990), the presence of phosphate glucose is detrimental to embryonic development at early cleavage because this compound enhances glycolysis, which, in turn, inhibits respiratory activity (Seshagiri and Bavister, 1991). Studies on the energy requirements of mammalian preimplantation embryos indicates that, in general, pyruvate or lactate are the best substrates during early development. Glucose utilization,

which is low at this stage, shows a steady increase with development until the morula or blastocyst stage (Chatot *et al*, 1990; Thompson *et al*, 1992). Surprisingly, the positive effect of the CZB medium on ovine embryos was not confirmed in the later stages of development, and no blastocysts cultured in the CZB medium completed an escape from the ZP. This is in contrast with the results of Ellington *et al* (1990) in cows, who found that 33% of bovine blastocysts hatched after 10 d culture in a CZB medium with an oviductal monolayer. Direct comparison between different species regarding the hatching process may not always be relevant. For example, it has been observed that the majority of mouse blasto-



Figs 3 and 4. 3. Hatching process of ovine blastocyst through the artificial opening after 6 d of culture in CZB medium. 4. Hatched blastocyst cultured in CZB medium for another 3 d underwent blastocoelic breakdown. Optical light magnification 250 x.

cysts fully hatch *in vitro*, while spontaneous hatching is rarely observed in human blastocysts (Bolton *et al*, 1989).

The morphology of the blastocysts obtained in the CZB cultures appeared normal and good expansion of the blastocoelic cavity with thinning of the ZP was observed *via* light microscopy. Our results also showed that these blastocysts completely hatched when transferred to TCM 199 medium supplemented with 10% FCS with or without oviductal cells. Moreover, the viability of the embryos cultured in this way to later develop *in vivo* was tested with good results with ovine embryos transferred into recipient ewes (Ledda *et al*, 1993). These data did not confirm a close correlation

between the ability of blastocysts to escape from the ZP and subsequent *in vivo* viability. The effects of TCM-199 may be due to the presence of serum, which demonstrated both inhibitory action at the first cleavage stage and stimulatory action at the compaction stage (Pinyopummintr and Bavister, 1991). However the use of TCM-199 medium with polyvinyl alcohol alone was also able to hatch ovine blastocysts (data not shown). It is unclear how TCM-199 medium improves blastocyst hatching. An explanation for the success of the TCM-199 medium might be that the balance of its other compounds, which were not present in CZB, was closer to the optimum for hatching ovine blastocysts than the simple medium

Table II. Effect of different culture systems on the hatching by ovine blastocysts from the ZP.

Culture systems in hatching process	Sources and number of blastocysts				Total number of hatching/hatched
	CZB (39)	CZB + SOEC (56)	TCM + 10% FCS + SOEC (52)	TCM + 10%FCS (6)	
TCM + 10% FCS	12 (8/8)	17 (12/11)	15 (9/8)		44 (29/27) ^d
TCM + 10% FCS + SOEC	8 (6/5)	13 (12/10)	12 (12/11)	3 (3/3)	36 (33/29) ^b
CZB	10 (1/0)	15 (2/0)	13 (2/0)		38 (5/0) ^c
CZB + SOEC	9 (3/0)	11 (3/0)	12 (4/0)	3 (1/0)	35 (11/10) ^a

Values in parentheses are number of blastocysts hatching/hatched; SOEC = sheep oviductal epithelial cells; within columns a *versus* b and c *versus* d, $p < 0.001$; b *versus* d, $p < 0.05$.

alone. On the other hand, vitamins, amino acids or other components such as inositol may have a key role in the regulation of embryo growth, as they have been shown to increase blastocyst expansion and enhance the process of blastocyst hatching in 3 different species (Kane and Bavister, 1988; Kane, 1989; Kane *et al*, 1992). These results are interesting in view of the role of inositol lipids in mediating the effect of growth factors on cell proliferation (Berridge, 1987).

The increased incidence of hatched blastocysts observed in microsurgically treated ovine morulae confirms previously determined data for pigs (Malter and Choen, 1989), mice and humans (Martin *et al*, 1991), where it was found that an incision in the zona enhances the ability of blastocysts to escape from the ZP. However, the efficiency of CZB medium to support this embryonic stage was very low because even after the embryos had hatched completely from the artificial hole, the blastocoelic cavity broke down.

Our experiments also demonstrated that the presence of oviductal epithelial cells in the TCM-199 medium enhanced the hatch-

ing process and increased, although not significantly, the number of ovine blastocysts hatched. The specific effect of co-culture on embryo development *in vitro* remains an open question. Whether the epithelial cells are actively involved in the removal of embryotoxic substances from the culture medium (Pampfer *et al*, 1990) or in the secretion of proteins with embryotrophic activity (Gandolfi *et al*, 1989) remain unresolved questions.

Although it is probable that both these mechanisms occur, at the moment there is only evidence of the effect for a growth factor on the hatching process. In fact, the addition of murine and human leukemia inhibitory factor to culture media enhanced the hatching process in mice and sheep, producing, in these species, a 4-fold increase in the number of blastocysts escaping from the ZP (Robertson *et al*, 1991; Fry *et al*, 1992).

In conclusion, these experiments suggested that CZB medium can better support *in vitro* embryonic development during the early cleavages but that TCM-199 medium was superior for the hatching pro-

cess when ovine embryos were cultured either with or without oviductal epithelial cells.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of VB Walker for the manuscript preparation. This work was supported by a grant from the National Council of Italy, Special Project RAISA, subproject No 3, paper No 2254.

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