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Original article

Coculture of ovine zygotes fertilized *in vivo* or *in vitro* and positive effect of CZB medium on the development of *in vitro* fertilized zygotes

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Summary — The development and quality of ovine zygotes derived from *in vivo* (IVOF) or *in vitro* fertilization (IVF) were compared after coculture on sheep oviductal cells. The same criteria were used to evaluate the coculture of IVF zygotes in CZB medium for 2 d followed by 199 medium for 6 d (CZB-199 coculture) or in 199 medium for 8 d (199 coculture). A higher overall developmental rate to blastocyst stages was obtained with IVOF (65.7%) than with IVF (23.2%) zygotes. More IVF zygotes reached blastocyst stages in CZB-199 (36.7%) than in 199 coculture (22.9%). The morphological aspect did not differ significantly between IVOF and IVF or between 199 and CZB-199 blastocysts. Histological examination revealed no significant difference in the pyknotic and mitotic indices and mean number of cells in the trophoblast and in the inner cell mass of hatched blastocysts between IVOF and IVF or between CZB-199 and 199 cocultures. According to criteria used in this study, the quality of blastocysts was equivalent, independently of fertilization or coculture systems. The use of CZB medium during the first cleavages increases the proportion of blastocysts.

ovine / embryo / development / culture

Résumé — Coculture d'œufs ovins fécondés *in vivo* ou *in vitro* et effet favorable du milieu CZB sur le développement d'œufs fécondés *in vitro*. *Le développement et la qualité d'embryons ovins obtenus après fécondation* in vivo *(IVOF) ou* in vitro *(IVF) ont été étudiés après coculture sur tapis de cellules épithéliales d'oviducte. Les mêmes critères ont été utilisés pour évaluer la coculture des œufs IVF pendant 8 j en présence de milieu 199 (coculture 199) ou CZB pendant les 2 premiers j (coculture CZB-199). Une proportion plus importante d'embryons IVOF (65,7%) que d'embryons IVF (23,2%) a atteint le stade blastocyste. Un plus grand nombre d'œufs IVF s'est développé jusqu'au stade blastocyste en coculture CZB-199 (36,7%) qu'en coculture 199 (22,9%). Les proportions de blastocystes classés morphologiquement en excellente, bonne, moyenne et mauvaise qualité en fin de culture ne sont pas significativement différentes quel que soit le type de fécondation (IVOF et IVF) ou de milieu de culture (CZB-199 et 199). L'examen histologique des blastocystes éclos ne révèle pas de différences*

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significatives entre les indices pycnotiques et mitotiques ainsi qu'entre le nombre des cellules de la masse cellulaire interne et celui des cellules du trophoblaste. Ainsi, les blastocystes sont de qualité équivalente, indépendamment du type de fécondation et du milieu utilisé en culture. En revanche, la présence du milieu CZB pendant les premières segmentations augmente la proportion de blastocystes obtenus après fécondation in vitro.

ovin / embryon / développement / culture

INTRODUCTION

Applying new techniques, such as gene transfer and nuclear transplantation, to farm animal species is still limited due to the reduced development and viability of embryos cultured *in vitro*. Long-term culture to the morula or blastocyst stages following *in vitro* fertilization (IVF) or other manipulations is advisable in order to assess embryonic viability prior to transfer, thus reducing the expenses related to recipient females.

The developmental block observed with the culture of sheep, goat and cattle embryos especially when synthetic defined media were used (Gandolfi and Moor, 1987; Rexroad and Powell, 1988; Eyestone and First, 1989; Sakkas *et al,* 1989; Thompson *et al,* 1989; McCaffrey *et al,* 1991; Prichard *et al,* 1992) could be overcome by temporarily incubating the embryos in oviducts from rabbits (Boland, 1984; Sirard *et al,* 1985), goats (Ryan *et al,* 1993) or sheep (Parrish *et al,* 1986; Crozet *et al,* 1987; Rexroad and Powell, 1988; Czlonkowska *et al,* 1991). Nevertheless, since Gandolfi and Moor (1987) demonstrated that a coculture with somatic cells could improve the development of ovine embryos, this system has been largely employed to obtain an adequate embryo development in ruminant species, after *in vivo* fertilization (IVOF) or IVF (Fukui *et al,* 1988; Rexroad and Powell, 1988/1991; Eyestone and First, 1989; Czlonkowska *et al,* 1991; Shamsuddin *et al,* 1993).

Even though a successful culture of *in vivo* fertilized ovine and bovine embryos in the synthetic oviduct fluid (SOF) medium under a reduced oxygen atmosphere (5%) has previously been reported by Tervit *et al* (1972), followed by Walker *et al* (1989), McLaughlin *et al* (1990) and Gardner *et al* (1994), only recently has a system without somatic cells been successfully employed in the culture of *in vitro* fertilized bovine (Kobayashi *et al,* 1994) and ovine (Watson *et al,* 1994) embryos. Furthermore, the studies of Kobayashi *et al* (1994) and Watson *et al* (1994) demonstrated that under an atmosphere of 5% CO_2 (20% O_2) a coculture system should be employed rather than culture without somatic cells.

Although TCM 199 is one of the most widely employed media for coculture, significant benefits of the use of a chemically defined simple medium like CZB has been demonstrated in the coculture of *in vivo* fertilized bovine (Ellington *et al,* 1990) and ovine embryos (Ledda *et al,* 1991). Furthermore, CZB-conditioned medium with either oviductal epithelial cells or Buffalo rat liver cells provided higher initial cleavage rates of *in vitro* fertilized bovine embryos than TCM 199 conditioned medium (Hernandez-Ledezma *et al,* 1993).

Little information is available on morphological aspects of *in vitro* fertilized sheep embryos developed in culture. Developmental rates to morula or blastocyst stages are usually the only criteria used for evaluating or for comparing culture systems. In this work, developmental rate and quality of ovine embryos was first compared

between *in vivo* and *in vitro* matured and fertilized zygotes, in a coculture system with sheep oviductal epithelial cells in TCM 199 medium. In addition, the effect of a coculture with sheep oviductal epithelial cells in CZB medium for the initial 48 h followed by coculture in TCM 199 medium for the remaining 6 d was compared to coculture in TCM 199 medium from the start.

In order to improve accuracy when analysing data from different culture conditions, we tried to establish a morphological assessment of the different phases of the blastocyst stage and to obtain separate counts of cells belonging to the trophoblast and to the ICM through histological study.

MATERIALS AND METHODS

Oocyte maturation

Sheep ovaries were obtained from a slaughterhouse and transported to the laboratory at 25–30°C in 0.9% NaCl solution containing 100 IU/ml penicillin and 50 μg/ml streptomycin. Aspiration of oocytes from 2–5 mm follicles, using a 5 ml syringe and a 20 gauge needle, was completed within 3 h after ovary collection. Liquid aspirated from follicles was transferred to a conical tube containing TCM 199-Hepes medium (Sigma, Saint Louis, MO) supplemented with 3% of foetal calf serum (FCS, Gibco, Grand Island, NY) and 10 IU/ml heparin (Sigma). Oocyte-cumulus complexes (OCCS) were recovered from the sediment, washed 3 times in maturation medium (TCM 199-Hepes + 10% FCS) and only cumulus-intact oocytes with evenly granulated cytoplasm were selected for maturation. Oocytes were matured in 4-well dishes (Nunc, Roskilde, Denmark), each well containing 1 ml of maturation medium supplemented with 110 μg/ml sodium pyruvate (Gibco), 0.1 μg/ml of pFSH and 0.02 μg/ml of pLH (Stimufol–Rhône-Merieux, Lyon, France), 1 μg/ml of oestradiol-17β (Sigma) and 10% of heat-inactivated FCS. Fresh granulosa cells were aspirated from non-atretic follicles with a diameter of 2–5 mm, centrifuged at 250 *g* for 4 min, washed twice and resuspended in TCM 199-Hepes and added to each maturation well at approximately 2 x 10^6 cells/ml. The OCCs were cultured for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

After maturation, the OCCs were briefly washed with a TCM 199-Hepes solution containing 150 IU/ml hyaluronidase (Sigma) to facilitate mechanical denudation of their cumulus cells by repeated pipetting into a thin diameter pipette.

Sperm capacitation and IVF

Frozen semen pooled from 4 different rams was thawed at 38°C for 20 s. A volume of 0.4 ml was applied to a Percoll (Pharmacia, Sweden) density gradient (45%/90%). After centrifugation for 20 min at 500 *g*, the supernatant was discarded and the sperm sediment was diluted with DM-Hepes medium supplemented with 20% of sheep oestrus serum (Crozet *et al*, 1987) to adjust the spermatozoa concentration to 1 x 10^7/ml. Sperm suspension (0.5 ml) was then capacitated for 60 min at 38.5°C in air.

For fertilization the spermatozoa were diluted to a final concentration of 1 x 10^6/ml in DM-Hepes supplemented with 20% of sheep oestrus serum and calcium lactate (pH 7.7), a fertilization medium suggested by Huneau and Crozet (1989). The denuded oocytes and the spermatozoa were incubated together for 16–17 h at 38.5°C in air.

Oviduct cell culture

The oviducts that provided cells for culture were collected at a slaughterhouse from sheep reproductive tracts whose ovaries showed recent ovulation; they were transported to the laboratory on ice. Procedures for preparing oviduct cell culture were similar to those described by Gandolfi and Moor (1987). Briefly, the oviducts were trimmed free of mesosalphinx and fimbriae and were flushed into a conical tube with culture medium (TCM 199 containing Earle's salts supplemented with 10% FCS). Flushed cells were allowed to sediment for 5–10 min and the supernatant was discarded. This procedure was repeated twice and the concentrated cell pellet was resuspended in 2 ml of culture medium. Cells were then distributed into 4-well dishes, cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air and used

for embryo culture 48 h later, when confluence reached 50–60%.

Embryo culture and assessment of morphology

The eggs were washed 3 times with fresh culture medium 16–17 h after mixing gametes and were transferred into the culture dishes containing the oviduct cells (30–40/well). The eggs were maintained in coculture with TCM 199 medium for 8 d at 38.5°C in a humidified atmosphere of 5% CO_2 in air. When the effect of CZB was studied, this medium was used for the first 48 h followed by TCM 199 for the remaining 6 d of coculture. CZB medium was prepared according to the formulation of Chatot *et al* (1989) and was supplemented with 5.0 mg/ml of BSA (Sigma) whereas TCM 199 medium was supplemented with 10% FCS. Embryos were transferred to a new monolayer of oviductal cells derived from the same primary culture which had medium renewed at 48 and 120 h after the beginning of culture.

Forty-eight hours after beginning of culture, embryos were scored for cleavage to ≥ 2 cells and those that were not developed were fixed in acetic acid/ethanol (1:3 v/v) for 24 h and stained with lacmoid (Sigma) in order to determine proportions of unfertilized and abnormally fertilized eggs (polyspermic eggs, presence of only one pronucleus or presence of non-decondensed sperm head).

All manipulations of gametes or embryos were performed in a warmed environment at 30°C. At the end of culture the embryos were assessed for morphological stage of development reached, *ie* expanded blastocyst (BX), hatching blastocyst (BH) and hatched blastocyst (BE). Furthermore, blastocysts were scored for morphological quality based on criteria of Elsden and Seidel (1982) into the following categories.

Excellent (grade I): blastocysts with spherical or slightly elongated aspect, distinct cellular outlines and embryonic structures (inner cell mass, trophoblast and blastocoel cavity), absence of intracytoplasmic vesicles, cellular debris or extruded cells;

Good (grade II): blastocysts with characteristics similar to those of *Excellent* but with some slight imperfections;

Fair (grade III): blastocysts with the same or a smaller size than *Excellent* or *Good* ones, slightly darker or clearer than normal, presence of intracytoplasmic vesicles, granulations on the cell surface, cellular debris in the blastocoel or extruded cells;

Poor (grade IV): blastocysts without distinct cellular outlines or embryonic structures, presence of pronounced imperfections.

The embryos that did not reach blastocyst stages were fixed and stained as described for non-developed zygotes in order to verify at which stage development was arrested.

Cell number determination

The number of nuclei and the presence of mitosis and pyknosis in hatched blastocysts were evaluated through histological examination. Hatched blastocysts were evaluated because they represented a greater number than hatching or expanded blastocysts thus allowing a separate analysis within grades considered for the morphological evaluation. When blastocysts contained one or more damaged sections due to histological procedures the exact number of cells could not be determined and they were discarded for the analysis of cell numbers. Some blastocysts (1 for IVOF, 2 for IVF-199 were 4 for CZB-199) were also lost due to problems not related to histological technique. Twelve blastocysts (9 hatched, 1 hatching and 2 expanded) from IVF-199 coculture were transferred by endoscopy into the uterus of 4 ewes, day 8 after oestrus. For this reason, 21 hatched blastocysts were available to histological analysis instead of 30.

Cell counts were performed after blastocysts had been fixed for 24 h in Bouin Holland containing 1.5% of acetic acid, double-embedded in agar-paraffin, cut into 10 μm serial sections and stained with haematoxylin-eosin. The nuclear state (pyknotic, interphasic or mitotic) was assessed and the number of trophoblastic and ICM cells was determined by microscopic examination (400 x).

Collection and culture of in vivo *fertilized embryos*

Mature ewes were treated for 14 d with a vaginal sponge (fluorogestone acetate, 40 mg) and were superovulated by 4 intramuscular injections of

decreasing doses (6, 5, 3 and 2 mg) of FSH (Stimufol) every 12 h (Cognie *et al*, 1985). Hormone stimulation started 24 h before sponge withdrawal. The ewes were hand-mated with fertile rams at 12 and 24 h after oestrus detection. One-cell embryos were recovered by flushing the oviducts with Dulbecco's phosphate-buffered saline (PBS) supplemented with 10% FCS, 24–36 h after mating.

After recovery, embryos were washed 3 times in culture medium and were placed in a 4-well dish on a confluent monolayer of oviductal epithelial cells. The monolayer was prepared in the same fashion as described above and the cells were recovered from ovulated sheep specifically prepared for this purpose.

Statistical analysis

Differences in the percentage of embryos reaching blastocyst stages and in the morphological aspect were determined by using Chi-square or Fisher's exact test. The number of cells in the trophoblast and in the ICM was submitted to a logarithmic transformation (SAS Stat Guide) before analysis by Student's *t*-test. The same test was employed to analyse the mitotic and pyknotic indices.

RESULTS

Zygotes that had 3 or more pronuclei or a non-decondensed sperm head in the cytoplasm after lacmoid staining were considered as abnormally fertilized and those that cleaved to at least the 2-cell stage at 48 h of culture were presumed to be normally fertilized. Cleavage rates to at least the 2-cell stage were 51 and 76% for IVM/IVF and *in vivo* fertilized zygotes, respectively.

A great proportion of embryos that failed to develop into blastocysts after *in vitro* maturation and IVF were fragmented, *ie* contained enucleated cytoplasmic spheres that could not be visually distinguished from normal blastomeres, until fixation and lacmoid staining. The proportion of fragmented embryos was significantly lower ($P \leq 0.05$) when culture was carried out in CZB-199 (21%) than in 199 (34%).

The overall developmental rate until blastocyst stages and the proportion of hatched blastocysts were higher in IVOF than in IVF embryos, after 8 d of coculture (table I). Coculture in CZB for 48 h followed by TCM 199 (CZB-199 coculture) provided a larger number of blastocysts and a greater proportion of hatched blastocysts (table II) than coculture in TCM 199 medium for 8 d (199 coculture).

Morphological aspects did not differ significantly ($P > 0.05$) either between IVOF and IVF or between TCM 199 and CZB-199 blastocysts, as indicated in tables III and IV, respectively. It is however noteworthy

Table I. Development to the blastocyst stage of IVOF or IVF ovine eggs cocultured with sheep oviductal cells for 8 d.

Fertilization system	*Number at ≥ 2 cell stage* [a]	*Developmental stage reached (%)*			
		BX	*BH*	*BE*	*BX + BH + BE*
IVOF	35	4 (11.4)	3 (8.6)	16 (45.7)[b]	23 (65.7)[b]
IVF	125	9 (7.2)	5 (4.0)	15 (12.0)[c]	29 (23.2)[c]

BX = expanded blastocyst; BH = hatching blastocyst; BE = hatched blastocyst. [a] Number of cleaved zygotes to at least the 2-cell stage at 48 h of culture. [b,c] Different superscripts within the same column indicate significant differences ($P < 0.05$).

that over 20% of blastocysts were of poor quality *(grade IV).*

Grade I and II hatched blastocysts were grouped because no significant differences were observed in the mean cell numbers or in the mitotic and pyknotic indices. Differences in at least one of these parameters were however observed between *grade I +*

Table II. Development to the blastocyst stage of *in vitro* fertilized ovine eggs cocultured on sheep oviductal cells for 8 d.

Coculture system	*Number at ≥ 2 cell stage* [a]	*Developmental stage reached (%)*			
		BX	*BH*	*BE*	*BX + BH + BE*
199	131	5 (3.8)	10 (7.6)	15 (11.4)[b]	30 (22.9)[b]
CZB-199	120	12 (10.0)	6 (5.0)	26 (21.7)[c]	44 (36.7)[c]

BX = expanded blastocyst; BH = hatching blastocyst; BE = hatched blastocyst. [a] Number of cleaved zygotes to at least the 2-cell stage at 48 h of culture. 199 = coculture with TCM 199 medium for 8 d. CZB-199 = coculture with CZB for 2 d followed by TCM 199 medium for 6 d. [b,c] Different superscripts within the same column indicate significant differences ($P < 0.05$).

Table III. Morphological aspect of ovine bastocysts obtained after IVOF or IVF and coculture with sheep oviductal cells for 8 d.

Fertilization system	*Number of blastocysts*	*Morphological grade (%)*		
		I + II	*III*	*IV*
IVOF	23	10 (43.5)	8 (34.8)	5 (21.7)
IVF	29	10 (34.5)	13 (44.8)	6 (20.7)

I = excellent; II = good; III = fair; IV = poor. Differences were not statistically significant ($P > 0.05$).

Table IV. Morphological aspect of ovine blastocysts obtained after IVF and coculture with sheep oviductal cells for 8 d.

Coculture system	*Number of blastocysts*	*Morphological grade (%)*		
		I + II	*III*	*IV*
199	30	8 (26.7)	14 (46.7)	8 (26.7)
CZB-199	44	16 (36.4)	15 (34.1)	13 (29.5)

I = excellent; II = good; III = fair; IV = poor. 199 = coculture with TCM 199 medium for 8 d. CZB-199 = coculture with CZB for 2 d followed by TCM 199 medium for 6 d. Differences were not statistically significant ($P > 0.05$).

II and *grade III* blastocysts thus justifying a separate analysis of these grades.

The proportions of *grade I, II and III* hatched blastocysts that had the number of cells determined were 85% (11/13), 76% (13/17) and 67% (14/21) for IVOF, IVF-199 and CZB-199, respectively. There were no significant differences in the mean number of cells in the ICM and in the trophoblast either between IVOF and IVF or between 199 and CZB-199 cocultures in *grade I + II* hatched blastocysts, as shown in tables V and VI, respectively. Nevertheless, the number of cells in the ICM tended to be higher ($P = 0.06$) in CZB-199 than in 199 coculture when *grade II* hatched blastocysts were compared separately. The same trend ($P = 0.07$) was observed for the trophoblastic cells in *grade III* blastocysts. Histological aspects of *grade* I hatched blastocysts can be observed in figure 1.

The proportions of *grade IV* hatched blastocysts that could be examined histologically were 100, 100 and 80% for IVOF, IVF-199 and CZB-199, respectively. The mean number of cells in this grade was 147 ($n = 3$), 143 ($n = 4$) and 141 ($n = 4$) under IVOF, IVF-199 and CZB-199, respectively. None of these *grade IV* blastocysts were taken into account for analysing the num-

Table V. Number of cells in hatched blastocysts obtained after IVOF or IVF and coculture with sheep oviductal cells for 8 d.

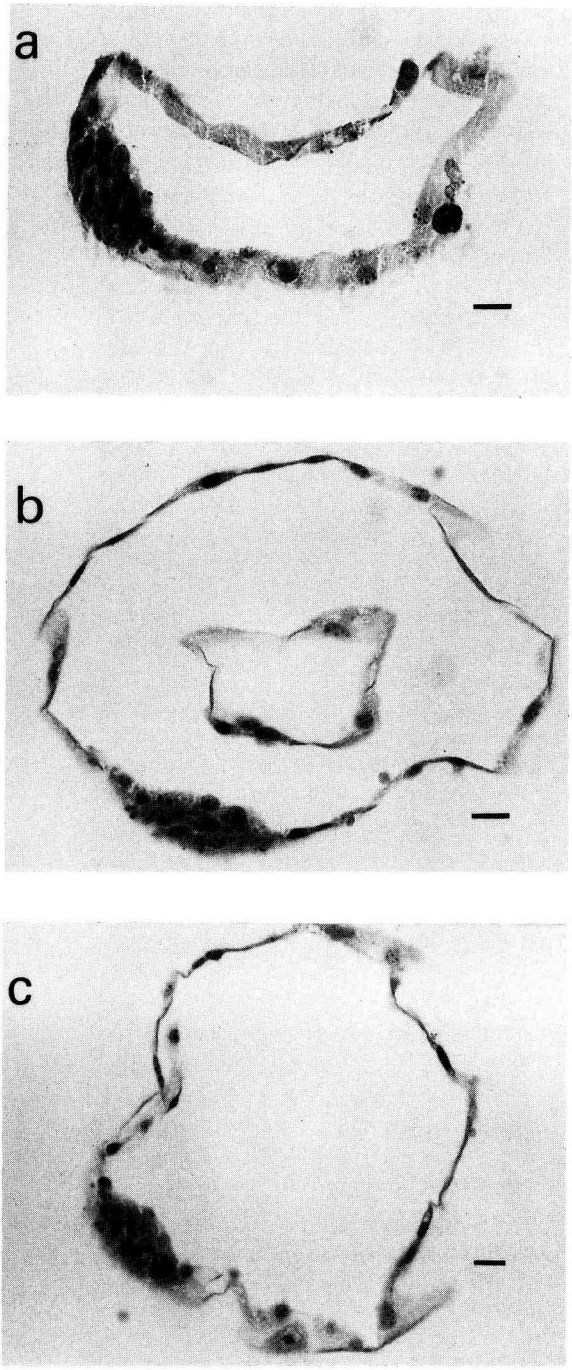
Morphological grade	*Fertilization system*	*Number of BE*	*Mean number of cells ± sd*			*Ratio ICM/total*
			TB	*ICM*	*TB + ICM*	
I + II	IVOF	9	434 ± 360	76 ± 30	510 ± 386	0.15
	IVF	7	398 ± 183	87 ± 30	485 ± 207	0.18
III	IVOF	2	283 ± 27	40 ± 16	323 ± 42	0.12
	IVF	6	174 ± 67	42 ± 7	216 ± 68	0.19

BE = hatched blastocyst; TB = trophoblast; ICM = inner cell mass. I = excellent; II = good; III = fair. Differences were not statistically significant between grade I + II embryos ($P > 0.05$). Data from grade III embryos were not compared statistically.

Table VI. Number of cells in hatched blastocysts obtained after IVF and coculture with sheep oviductal cells with CZB medium for 2 d followed by TCM 199 medium for 6 d.

Morphological grade	*Number of blastocysts*	*Mean number of cells ± sd*			*Ratio ICM/total*
		TB	*ICM*	*ICM + TB*	
I + II	10	511 ± 161	114 ± 38	625 ± 175	0.18
III	4	259 ± 72	41 ± 13	300 ± 79	0.14

TB = trophoblast; ICM = inner cell mass; I = excellent; II = good; III = fair. Significant differences were not observed ($P > 0.05$) between 199-CZB and 199 coculture (compared with data presented as IVF in table V).



ber of cells because they presented zero or less than 20 cells in the ICM, according to histological examination.

The pyknotic index of the trophoblast and of the ICM cells in *grade I + II* hatched blastocysts did not differ ($P > 0.05$) between IVOF and IVF (table VII). Although no significant difference was observed in the pyknotic index of the trophoblast and of the ICM cells in *grade I + II* and *grade III* hatched blastocysts between 199 and CZB-199 cocultures, the pyknotic index of trophoblastic cells tended to be higher ($P = 0.07$) in 199 (7.7) than in CZB-199 (4.2) when *grade II* blastocysts were compared separately. In contrast, the pyknotic index of trophoblastic cells in *grade III* blastocysts tended to be higher ($P = 0.06$) in CZB-199 (6.7) than in 199 coculture (4.1).

The average mitotic index was low in the ICM of hatched blastocysts under all conditions evaluated (tables VII and VIII). Due to the absence of mitotic cells in several of the hatched blastocysts examined, the mitotic index of the ICM was not compared statistically. Mitosis was always observed in trophoblastic cells of all blastocysts examined. The mitotic index of trophoblastic cells was not significantly different either between IVOF and IVF or between TCM 199 and CZB-TCM 199 hatched blastocysts (tables VII and VIII).

Data on *grade III* hatched blastocysts were not compared statistically between IVOF and IVF because few ($n = 2$) IVOF hatched blastocysts could be examined.

Fig 1. Histological section of *grade I* hatched ovine blastocysts obtained in coculture for 8 d with sheep oviductal cells (haematoxylin-eosin staining). After IVOF (**a**) or IVF (**b**) and coculture with 199 medium; after IVF and coculture with CZB medium for 2 d and 199 for 6 d (**c**). Bar = 25 μm.

Table VII. Mitotic and pyknotic indices of hatched blastocysts obtained after IVOF or IVF and coculture with sheep oviductal cells for 8 d.

Morphological grade	*Fertilization system*	*Number of blastocysts*	*Mitotic index*		*Pyknotic index*	
			ICM	*TB*	*ICM*	*TB*
I + II	IVOF	9	0.6	1.6	9.1	5.2
	IVF	7	0.7	1.4	10.0	5.5
III	IVOF	2	1.3	1.1	8.8	8.1
	IVF	6	2.0	1.5	11.2	4.1

I = excellent; II = good; III = fair; ICM = inner cell mass; TB = trophoblast. Data concerning *grade III* embryos and the mitotic index of ICM in *grade I + II* embryos were not compared statistically. Differences were not statistically significant between IVOF and IVF *grade I + II* embryos ($P > 0.05$).

Table VIII. Mitotic and pyknotic index of hatched blastocysts obtained after IVF and coculture with sheep oviductal cell monolayers with CZB medium for 2 d followed by TCM 199 medium for 6 d.

Morphological grade	*Number of blastocysts*	*Mitotic index*		*Pyknotic index*	
		ICM	*TB*	*ICM*	*TB*
I + II	10	2.1	1.8	8.6	3.6
III	4	0.6	1.5	11.6	6.7

I = excellent; II = good; III = fair; ICM = inner cell mass; TB = trophoblast. Significant differences were not observed ($P > 0.05$) between 199-CZB and 199 coculture (compared with data presented as IVF in table VII).

DISCUSSION

The results of this study show that the morphological aspect and numbers of cells of blastocysts obtained in coculture after IVOF were comparable to those of blastocysts obtained after IVF. More IVOF zygotes developed to blastocyst stages, underlining the lower ability of IVF zygotes to support *in vitro* culture conditions.

The favourable effect of CZB, which does not contain glucose, on blastocyst formation indicates that ovine embryos can utilize other energetic sources than glucose for their first cleavages. Since Thompson *et al* (1991) observed a significant increase on glucose utilization from 8-cell-stage ovine embryos, zygotes cocultured in CZB medium were moved to 199 medium containing glucose in order to fulfil their energetic requirements at 48 h of culture (corresponding to 65 h after *in vitro* insemination).

High glucose concentrations, like those present in TCM 199 medium, have been considered detrimental to *in vitro* development of mouse (Chatot *et al*, 1989), ham-

ster (Schini and Bavister, 1988), bovine (Takahashi and First, 1992) and ovine embryos (Thompson *et al,* 1992). Nevertheless, when its concentration ranges from 0 to 3 mM in the presence of pyruvate and/or lactate, glucose does not seem to affect ovine development, as already reported by Thompson *et al* (1992). Since pyruvate and lactate are not present in TCM 199 medium, embryos are basically dependent on glucose and amino acids as sources of energy. This may have contributed to the lower developmental rate observed when this medium was utilized throughout the culture.

Histological studies are time-consuming but allowed us to differentiate ICM from trophoblastic cells in addition to observing the presence of mitosis and pyknosis. More rapid procedures which allow accurate cell number determination after differential labelling of trophoblastic and ICM nuclei using 2 polynucleotide-specific fluorochromes (Handyside and Hunter, 1984; Papaioannou and Ebert, 1988) could have been used rather than histological analysis. The present study is, to our knowledge, the first to take into account the number of cells in the ICM and in the trophoblast to be carried out on ovine-hatched blastocysts obtained by a fully *in vitro* system, and so histological analysis was preferred because reference data are available from evaluations previously performed on several stages of *in vivo* fertilized ovine embryos (Wintenberger-Torrès, 1967; Wintenberger-Torrès and Sévellec, 1987). A comparison between histological and differential ICM/trophoblast cell-counting would certainly be important. Nevertheless, fluorescent counting is likely to be more accurate if applied to less advanced blastocysts (hatching and expanded) since determination of ICM cells may be impaired by packing cells in a whole-mount observation. The considerable variation in the number of cells observed in our study among embryos of the same morphological aspect and stage was also reported in ovine (Wintenberger-Torrès, 1967; Wintenberger-Torrès and Sévellec, 1987) and porcine (Papaioannou and Ebert, 1988) embryos derived *in vivo.* Recently, variations in cell numbers observed at various developmental stages in bovine embryos were mainly attributed to a difference in the developmental rate between male and female embryos (Xu *et al,* 1992a).

Despite the great variation of cell numbers observed even within the same morphological grade, histological analysis confirmed differences established by morphological evaluation except between *grades I* and *II.* Differences between these classes could have been observed if a greater number of embryos had been available. In addition, morphological classification avoided biased comparisons of cell numbers and made it possible to have differential losses during histological processing of embryos.

The mean ICM/total number of cells ratio observed in hatched blastocysts was lower than that observed in expanded blastocysts (0.29) which were evaluated after 6 d of coculture, in a preliminary experiment (data not shown). This is however a normal event when the blastocysts develop beyond the expanded stage as already reported for *in vivo* ovine embryos (Wintenberger-Torrès, 1967) and porcine embryos (Papaioannou and Ebert, 1988), and for bovine embryos fertilized *in vivo* and *in vitro* and cultured *in vitro* (Marquant-Le Guienne *et al,* 1989).

It is well known that culturing delays development even in embryos fertilized *in vivo* (Rexroad and Powell, 1991; Walker *et al,* 1992). It has been reported that ovine embryos fertilized *in vivo* have a higher potential to develop in culture when they are collected at more advanced stages (Wright *et al,* 1976; Peters *et al,* 1977; Lindner *et al,* 1979). Thus, only presumptive 1-cell *in vivo* fertilized zygotes were used in this study in order to have a more accu-

rate comparison since they should be more similar to those matured and fertilized *in vitro,* with respect to developmental ability, than embryos at more advanced stages.

Since embryonic viability tends to diminish with longer culture times (Tervit and Rowson, 1974; Gandolfi and Moor, 1987; Rexroad and Powell, 1991; Walker *et al,* 1989/1992), the high rate of poor quality blastocysts, the pyknotic and mitotic indices observed in the ICM were not surprising. Ellington *et al* (1990) also reported a low mitotic index among *in vivo* fertilized bovine embryos developed in coculture with simple or complex media. Furthermore, it was demonstrated that the mitotic index normally decreases from morula to hatched blastocysts (Iwasaki and Nakahara, 1990; Xu *et al,* 1992b) when bovine embryos were cultured *in vitro* or transferred to intermediate recipients. Even though the mitotic index in the ICM of bovine-hatched blastocysts analysed histologically by Marquant-Le Guienne *et al* (1989) was higher than in the present study, a comparable and a higher pyknotic index was detected for *in vitro* and *in vivo* fertilized bovine embryos respectively.

In vitro culture is usually associated with several developmental abnormalities such as cytoplasmic fragmentation and reduced number of cells per blastocyst (Walker *et al,* 1992). Distinction between viable and non-viable embryos is difficult due to cytoplasmic fragmentation as previously observed in cultured ovine embryos derived from *in vivo* fertilization (Tervit *et al,* 1972; Gandolfi and Moor, 1987; McGinnis and Youngs, 1992; Walker *et al,* 1992) and even in ovine embryos fully developed *in vivo* (Killen and Moore, 1971). Furthermore, Moor and Trounson (1977) observed that over 30% of the embryos obtained from *in vitro* matured sheep oocytes that were transferred to the oviducts of inseminated recipient ewes for 7 d were fragmented.

The absence of glucose in CZB medium probably contributed to the reduction of fragmentation observed in CZB-199 cocultured embryos. Indeed, McGinnis and Youngs (1992) observed that cytoplasmic fragmentation increased when CZB medium was supplemented with glucose in the culture of *in vivo* fertilized ovine zygotes. Walker *et al* (1992) could not establish the influence of a specific component of SOF medium on the incidence of fragmentation of ovine zygotes. They observed however a high incidence of fragmentation when embryos were cultured in microdrops of medium under paraffin oil or when culture was performed in MEM medium, which contains a higher glucose concentration than SOF medium.

Coculture has been largely employed for culturing mammalian embryos and several hypotheses that have been formulated to explain how these cells provide beneficial effects to embryonic development are now questionable. Production of specific or non-specific factors that stimulate embryo development does not seem not to be the most plausible cause since culture without somatic cells is feasible.

Deletion of components of culture media that are unfavourable for embryo development (Bongso *et al,* 1991; Pinyopummintr and Bavister, 1991), such as ammonium produced by spontaneous or embryo breakdown of amino acids (Gardner and Lane, 1993) or changes in the physico-chemical environment (pH, gas tension) provided by somatic cells (Bavister, 1988), remain the best explanations for the beneficial effects of coculture.

All recipient females, after transfer of blastocysts obtained in IVF-199 system, were pregnant and were allowed to go to term. From the 4 ewes, 3 male and 3 female live lambs were born (50%). This results confirm the viability of IVM/IVF zygotes cocultured in 199 medium but lacks importance because no comparisons were performed with blastocysts obtained after CZB-199 coculture.

Even if the reasons for the better development in CZB coculture were not elucidated in this study, it is evident that favourable effects of coculture are dependent on medium composition. Furthermore, when comparisons between coculture and culture without somatic cells were carried out in different oxygen tensions (Kobayashi *et al,* 1994; Watson *et al,* 1994), coculture was the most effective system in a 20% O_2 atmosphere. In contrast, culture in synthetic media like CZB (McGinnis and Youngs, 1992) or SOF (McLaughlin *et al,* 1990; Gardner *et al,* 1994; Watson *et al,* 1994) has been successfully carried out in an atmosphere with 5% O_2.

Embryo culture is now being oriented toward better defined systems without the use of somatic cells and complex media thus allowing a better study of the requirements of embryo development. For instance, comparisons of developmental rates and quality of blastocysts obtained with CZB-199 coculture and culture in SOF medium are being run in our laboratory.

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