Original article

Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems

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Summary — A Vero cell line was used for coculture of bovine in vitro fertilized eggs up to blastocyst stage in comparison with bovine oviductal epithelial cells (BOEC) in two culture systems: monolayers or microdrops. Inseminated oocytes cocultured for 7 days with Vero cells in microdrops resulted in a significantly higher blastocyst rate compared to BOEC (29.5% vs 21.1%, respectively; $P < 0.01$). This difference was not significant in the monolayer coculture system although the blastocyst rate tended to be higher with Vero than with BOEC monolayers (27% vs 22.3%, respectively). Interestingly, the coefficient of variation between replicates was lower in both Vero cell groups than in BOEC groups indicating that Vero cells may help reduce variability. Medium conditioned by Vero cells partly supported embryo development compared to coculture itself (14.6% vs 26.5%, respectively; $P < 0.01$). Blastocysts developed on Vero cells contained significantly more cells (142 ± 39) than those developed on BOEC (88.8 ± 32.8, $P < 0.001$). Viability of blastocysts developed on Vero cells was evaluated by single transfer to 26 recipient heifers. Confirmed pregnancy rate after 3 months was 58%, demonstrating their high viability.

bovine / embryo / coculture / IVF / blastocyst

Résumé — Apport des cellules Vero pour le développement en coculture des embryons bovins issus de FIV. Les cellules Vero ont été utilisées pour la coculture jusqu'au stade blastocyste des ovocytes bovins fécondés in vitro, en comparaison avec des cellules tubaires (BOEC), dans deux systèmes de culture différents : monocouche ou microgoutte. En microgouttes, le pourcentage de blastocystes obtenus à j7 est significativement plus élevé sur cellules Vero que sur BOEC (29,5 contre 21,1 %, respectivement ; $p < 0,01$). En monocouche, la différence n'est pas significative, mais reste en faveur des cellules Vero (27 contre 22,3 %, respectivement). En présence de cellules Vero, nous

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observons un plus faible coefficient de variation entre répliques. Ce système de coculture peut contribuer à réduire la variabilité de la production d'embryons in vitro. Le milieu conditionné par les cellules Vero permet un développement plus limité comparé au système de coculture (14,6 % de blastocystes contre 26,5 %, respectivement; $p < 0,01$) à j7. Les blastocystes développés sur cellules Vero ont significativement plus de cellules (142 ± 39) que ceux développés sur BOEC (88,8 ± 32,8; $p < 0,001$). La viabilité des blastocystes développés sur cellules Vero a été évaluée par transplantation dans 26 génisses receveuses. À 3 mois, le taux de gestation était de 58 %, confirmant la bonne viabilité de ces embryons.

bovin / embryon / coculture / FIV / blastocyste

INTRODUCTION

For several mammalian species, the in vitro development of zygotes up to the blastocyst stage is limited and frequently stopped at the time of genomic activation (Menezo et al, 1992). In cattle, to improve in vitro development of zygotes, a variety of coculture systems have been developed using different cell types such as fibroblasts (Kusan and Wright, 1982), trophoblastic vesicles (Camous et al, 1984; Heyman and Menezo, 1987), cumulus cells (Kajihara et al, 1987), granulosa cells (Rexroad, 1989) or bovine oviductal epithelial cells (Gandolfi and Moor, 1987; Eyestone et al, 1987). All these feeder cells used in coculture were able to improve in vitro development of bovine zygotes compared to a medium devoid of cells. Nevertheless it has been shown recently that in vitro fertilized bovine embryos can be successfully obtained in defined culture medium such as synthetic oviduct fluid (SOF) medium, but low O_2 tension is required (Trounson et al, 1994; Carolan et al, 1995).

The most commonly used coculture system for in vitro production of bovine blastocysts after in vitro maturation and fertilization of oocytes is bovine oviductal epithelial cells (BOEC). BOEC monolayers are prepared from fresh oviducts recovered at the slaughterhouse and have to be established for each in vitro fertilization (IVF) experiment. Furthermore even if the cow's oestrous cycle stage does not influence the ability of such cells to support development (Thibodeaux et al, 1992),

BOEC monolayers prepared from different animals could be a source of variation between replicate experiments and a source of contamination because of the unknown origin of the oviducts. It was shown that BOEC are able to secrete a variety of molecules including growth factors into the culture medium (Gandolfi et al, 1992; Eriksen et al, 1993). However, the mechanism of this action is still unclear. It seems that the embryotrophic or embryo-regulating factors are neither oviduct specific, hormone-dependent (Papaioannou and Ebert, 1986) nor species specific (Boland, 1984). Rather, they seem to be dependent upon the metabolism of transport epithelial cells. Other epithelial cell types particularly from liver (buffalo rat liver cells, BRL) and kidney (Mardin Darby bovine kidney cells or MDBK and Vero cells), efficiently support the development of mouse, bovine and human embryos (Ouhibi et al, 1990; Menezo et al, 1990; Voelkel and Hu, 1992; Myers et al, 1994; Reed et al, 1996). Vero is a well-defined, established cell line of green monkey (*Cercopithecus aethiops*) kidney epithelial cells. This cell line constitutes a sanitary safe support as it is highly controlled for known viruses and other contaminants (Menezo et al, 1990). These cells are used in the preparation of poliomyelitis and rabies vaccines for humans (legal authorization no AMM 315-070-8 and 317-070-8, respectively). The Vero cell line is easy to handle and to freeze and can retain the ability to provide the favourable conditions for embryo development over many passages

in humans at least (Menezo et al, 1992). Furthermore cryostorage of such a cell line allows better standardisation of the coculture conditions, as the same source of cells can be used over a long period.

The objective of this study was to investigate the use of Vero cells in coculture systems for the development of bovine in vitro matured and fertilized oocytes in comparison with control presumptive zygotes cultured on bovine oviductal cells. Monolayer and microdrop systems were established with both BOEC and Vero cells. As the preparation of BOEC is inconvenient and is a source of variation for the in vitro development, we hypothesized that the use of Vero cells could reduce the variation in bovine blastocyst formation in vitro.

MATERIALS AND METHOD

In vitro maturation and fertilization of oocytes

Bovine ovaries were obtained from a slaughterhouse and transported within 3 h to the laboratory, in sterile phosphate-buffered saline (PBS) maintained at 30 °C. Small follicles (2-8 mm) were punctured using a 19G needle and the selected intact cumulus oocyte complexes (COC) were washed three times in Hepes-buffered M199 medium (GIBCO). In vitro maturation (IVM) was performed in M199 medium (GIBCO) supplemented with 10% foetal calf serum (FCS), 10 μg FSH-LH/mL and 1 μg oestradiol 17-β/mL. The COCs were incubated in 0.5 mL of maturation medium in four-well Nunc plates on a bovine granulosa cell monolayer for 24 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. After this period, cumulus-expanded oocytes were in vitro inseminated with frozen-thawed sperm.

The same batch of frozen semen from one bull was used throughout all the experiments. Modified Tyrode's medium (TALP) was used for capacitation and fertilization (Parrish et al, 1986). After thawing, motile sperm were selected by the swim up technique as previously described by Marquant-Le Guienne et al (1989), concentrated by centrifugation and resuspended to a final concentration of 1×10^6 cells/mL in the fertilization medium containing 10 μg/mL of heparin. Groups of 20-30 oocytes in each well were co-incubated with sperm for 18 h at 39 °C.

For each replicate, after IVF, the zygotes were pooled and cumulus cells were removed mechanically by pipetting. Denuded presumptive zygotes were alloted to the different culture conditions: coculture with Vero cells versus BOEC in monolayers or microdrops, or control coculture versus Vero cell conditioned medium.

Coculture of inseminated oocytes

Preparation of Vero cell monolayers and microdrops

A frozen sample of the established Vero cell line, checked to be virus-free, was provided by Rhone-Mérieux (Lyon, France) and multiplied according to the protocol previously described by Menezo et al (1990). In brief, a culture was established from the frozen cryotube containing $2-3 \times 10^6$ cells. The 25 cm^2 culture flask (FALCON), the four-well dish (NUNC) and the microdrops (50 μL) were seeded at the concentration of 1×10^6 cells per flask, 1×10^5 cells per well or 1×10^3 cells per microdrop, respectively. The B_2 medium supplemented with 10% FCS plus 1% fungizone (GIBCO) and 0.5% penicillin-streptomycin (GIBCO) was used as cell-growth culture medium at 39 °C under 5% CO_2 in air. At confluency, the wells and the microdrops were ready for the coculture of presumptive zygotes. The flask was trypsinised for sub-passages to prepare other monolayers and microdrops. Four sub-cultures from the flask cultures were carried out and at the fourth one only the new plates and microdrops were seeded and the remaining cells were frozen. The technical procedure for cell freezing and thawing was the same as that described by Ouhibi et al (1990). Each cryotube contained $2-3 \times 10^6$ cells approximately in B_2 medium supplemented with 50% FCS and 10% dimethyl sulphoxide (DMSO). Freezing was carried out using a programmable freezer (Minicool, Air Liquide, France). The cooling rate was 1 °C/min from +2 °C to –10 °C, then 2 °C/min from –10°C to –30 °C and then 10 °C/min to –150 °C. Finally, the frozen cells were stored in liquid nitrogen.

Preparation of BOEC monolayers or microdrops

Bovine oviducts were collected from the abattoir and placed in sterile PBS to be transported on ice to the laboratory. The primary cultures of BOEC were prepared every week and no trypsinisation was carried out. The BOEC monolayers were prepared as follows: 1-2 mL of Hank's balanced salt solution (HBSS) without Ca^{+2} and Mg^{+2} was injected into the lumen of the oviduct ligated at both ends. After 15 min, the medium containing the cells was removed and centrifuged at 2 000 rpm for 5 min. The supernatant was eliminated and the pellet resuspended into 2 mL of fresh culture medium consisting of M199 supplemented with 10% FCS, 2.2 g/L of sodium bicarbonate plus 1% glutamine (200 mM), 1% serotonin (1 mg/mL), 1% fungizone (GIBCO) and 0.5% penicillin-streptomycin (GIBCO). 500 µL per well were distributed into Nunc plates. The culture was maintained at 39 °C with 5% CO_2 in air and the medium was renewed every 48 h. Cell monolayers were used at confluency (ie, 3 to 5 days).

Microdrops of 50 µL of B_2 medium under mineral oil were seeded with a fresh suspension of BOEC one day before starting coculture.

Comparison of coculture systems using Vero cells or BOEC

On day 1 of the coculture, the medium of the monolayer (Vero or BOEC) was removed and replaced with fresh B_2 medium supplemented with 10% FCS. This was later used as the embryo culture medium.

The presumptive zygotes were then allotted to two coculture system groups: monolayers of Vero cells or BOEC in four-well plates; 20 to 30 zygotes per well in 500 µL of medium; microdrops of Vero cells or BOEC; 10 to 20 zygotes per drop of 50 µL covered by mineral oil.

Both groups of coculture were carried out under the same conditions inside the same incubator at 39 °C under a humidified atmosphere of 5% CO_2 in air for 7 days. During this time the culture medium was not renewed. After 36 h of coculture the embryos in each group were rapidly evaluated for cleavage. On day 7 of coculture the number of blastocysts obtained was recorded and the morphological evaluation was carried out using a stereomicroscope. At this time, a comparison of development rates between the groups was made.

Preparation of conditioned medium on Vero cells

Vero cells were used to prepare conditioned medium. At confluence in the flask, fresh B_2 medium supplemented with 10% FCS was conditioned on the monolayer of Vero cells for 48 h. The medium was then removed, aliquoted in small samples and stored frozen at –20 °C until use. For each experiment a sample of conditioned medium was thawed and 50 µL drops of medium were placed under mineral oil and equilibrated overnight at 39 °C and 5% CO_2 in air. Ten to 20 presumptive zygotes were alloted per drop and incubated at 39 °C in a humidified atmosphere of 5% CO_2 in air for 7 days. The efficiency of such a conditioned medium in supporting development of presumptive zygotes was compared to that of Vero cell monolayers and to that of control B_2 medium + FCS which was not conditioned.

Evaluation of cell number in blastocysts

On day 7 of coculture, some of the blastocysts developed on Vero cells or BOEC monolayers were used for evaluation of cell number. Individual blastocysts were fixed in a solution of 2.5% paraformaldehyde then treated with 0.5 M NaOH for permeability and stained with propidium iodide for 15 min at 37 °C to label the nuclei. Each embryo was mounted on a slide for observation using a laser scan confocal microscope (LSM Zeiss) Optical sections of 4 to 5 µm were made and the exact number of nuclei for each blastocyst was counted through all the optical sections of the embryo.

Statistical analysis

Cleavage and blastocyst formation rates were compared between groups by Chi square test (X^2) and blastocyst cell number by Student's-t test.

RESULTS

The development rate of the inseminated oocytes was compared according to cell

types (Vero vs BOEC) in the two coculture systems (monolayer or microdrop). After 36 h of coculture 88.5% and 90.1% of the eggs were cleaved on Vero cell monolayers and microdrops, compared to 91% and 86.1% on BOEC monolayers and microdrops, respectively. There was no significant difference between groups (table I). On day 7, the blastocyst rate was 27% on Vero cell monolayers compared to 22.3% on BOEC monolayers. Although blastocyst formation tended to be higher on Vero cells, the difference was not significant between the groups (table I). Interestingly, the coefficient of variation of blastocyst rate between replicate experiments was two times lower (0.2) in the Vero than in the BOEC monolayer system (0.4) (fig 1).

When co-culture was performed in microdrops, the rate of blastocyst formation was significantly higher ($P < 0.01$) on Vero cells than on BOEC (29.5% vs 21.1%, respectively; table I) and the coefficient of variation between replicate experiments was four times lower (0.05) on Vero than on BOEC microdrops (0.20) (fig 2).

Table I. In vitro development of embryos in coculture using Vero cells vs BOEC.

Culture System	Cells	No of replicates	Presumptive zygotes	Cleaved (%)	Blastocysts (%)
Monolayer	Vero	11	474	417/474 (88.5%)	127/474 (27%)
Monolayer	BOEC	11	444	404/444 (91%)	100/444 (22.3%)
Microdrop	Vero	5	610	550/610 (90.1%)	180/610[a] (29.5%)
Microdrop	BOEC	5	549	473/549 (86.1%)	116/549[b] (21.1%)

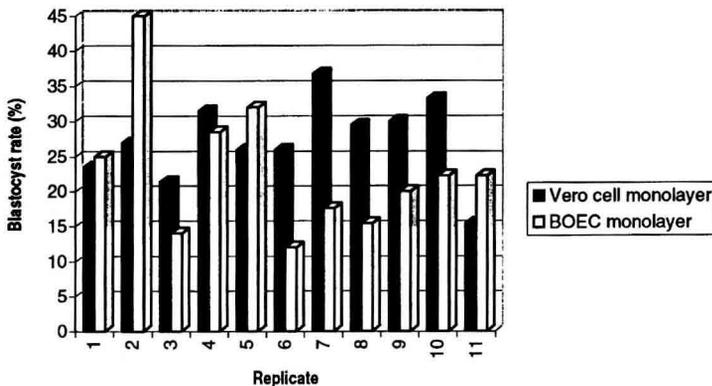[a, b] values differ significantly, X^2 test, $P < 0.01$.

Fig 1. In vitro development of bovine embryos to the blastocyst stage following coculture on Vero cell or BOEC monolayers. Coefficient of variation is 0.2 in Vero cells and 0.4 in BOEC.
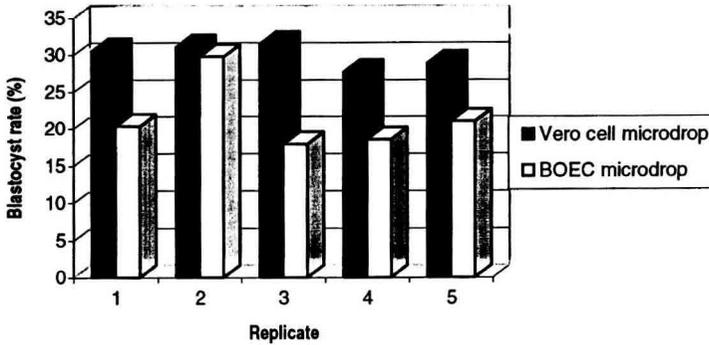
Fig 2. In vitro development of bovine embryos to the blastocyst stage following coculture on Vero cell or BOEC microdrops. Coefficient of variation is 0.05 in Vero cells and 0.2 in BOEC.

Blastocysts developed on Vero cells had a significantly higher number of cells than those developed on BOEC during the same period of culture (7 days). The mean number of cells (mean ± SD) as assessed by nuclei counting in blastocysts was $\bar{X} = 142 \pm 39$ ($n = 29$ embryos) on Vero cells compared to $\bar{X} = 88.8 \pm 32.8$ nuclei ($n = 19$ embryos) for those developed on BOEC, which was significantly different ($P < 0.001$). Blastocyst formation appeared to occur earlier in coculture with Vero cells as most of the blastocysts were observed on day 6 (data not shown).

In vitro development of zygotes in microdrops of conditioned medium was compared to that on Vero monolayers or to that in B_2 + FCS alone (not conditioned). Results are indicated in table II. The cleavage rate was not significantly different between groups but the blastocyst rate in conditioned medium was 14.6% (33/226) compared to 26.5% (61/230) in coculture ($P < 0.01$) and less than 1% (1/132; $P < 0.001$) in medium on its own. This indicates that conditioned medium only partly reproduced the beneficial effects of Vero cell coculture for bovine zygotes up to the blastocyst stage.

Table II. In vitro development of zygotes in medium conditioned by Vero cells.

Group	No of replicate experiments	Presumptive zygotes	Cleaved (%)	Blastocysts (%)
Vero Cell Monolayer	6	230	198/230 (86.1%)	61/230 (26.5%)[a]
Conditioned Medium	6	226	183/226 (81%)	33/226 (14.6%)[b]
B_2 +FCS (control)	3	132	99/132 (75%)	1/132 (0.7%)[c]

[a,b] percentages differ significantly, X^2 test, $P < 0.01$.
[a,c; b,c] percentages differ significantly, X^2 test, $P < 0.001$

Some day seven blastocysts developed on Vero cell were transferred to 26 synchronized recipient heifers (one embryo per recipient). Seventeen recipients were pregnant at day 35 (65.4%) and 15 were continuing pregnancy after 3 months (58%). This result confirms the high viability of IVF blastocysts developed in Vero cell cocultures.

DISCUSSION

In this study the coculture of bovine IVF presumptive zygotes on Vero cell microdrops resulted in a significantly higher blastocyst yield than coculture on BOEC. Furthermore, the use of Vero cells contributed to reduce the variability observed in development rates within replicate experiments as demonstrated by the lower coefficient of variation in both groups containing Vero cells compared to those with BOEC.

In our experiments the overall blastocyst yield in presence of Vero cells was higher than that obtained by Myers et al (1994). These authors reported a 30% blastocyst rate, but their rate is expressed as a ratio to cleaved eggs (2-4 cell) and not to inseminated oocytes as in our study. Recently, Maeda et al (1996) also used this cell line for coculture and they reported only 11% blastocyst formation. However, they used a protein and amino acid free simple human tubal fluid (HTF) medium instead of B_2 medium, which is considered to be more appropriate for coculture of bovine embryos than other media (Hasler et al, 1996).

Concerning the two coculture systems used (monolayer vs microdrop), we observed a slightly better blastocyst formation when zygotes were cocultured on Vero cells in the microdrop as compared with the monolayer system (29.5% vs 27%, respectively). This could be related to the embryo density in the culture medium since good results were reported culturing one embryo

per µL of medium under mineral oil (Ferry et al, 1994). It was suggested, at least for sheep and cattle embryos, that there is a cooperative interaction among the embryos and this interaction could be mediated by autocrine and/or paracrine factors. Also, the volume of culture medium and the number of embryos per unit volume could influence this interaction (Ferry et al, 1994; Gardner et al, 1994; Massip et al, 1995).

Other established cell lines that retain the ability to provide favourable conditions for embryo development over many passages have been investigated. The development rate obtained with BRL cells (Reed et al, 1996) or MDBK cells (Myers et al, 1994) also demonstrates that such cells are able to support development of bovine embryos but with a lower efficiency than Vero cells, at least when compared with this study.

One advantage of using the Vero cell line for coculture instead of BOEC is the reduction of variability between replicate experiments. Another advantage is that the same line from a frozen sample can be used over a long period unlike BOEC, whose primary cultures need to be prepared each week from unknown slaughterhouse oviducts which are a source of uncontrollable variation (Xu et al, 1992).

We also investigated the ability of a medium conditioned by Vero cells to support embryonic development and we observed significantly more blastocysts formed in conditioned medium than in the medium on its own but the blastocyst rate was still lower than in coculture with Vero cells. A conditioned medium has some advantages over that of a coculture system, eg, it may be stored frozen and the same batch of medium can be used for several replicates (Eyestone et al, 1991). It has been said that feeder cells may function by secreting embryotrophic factors and/or by modifying the culture medium by a detoxification process (Menezo et al, 1990; Kane et al, 1992). Chen et al (1994) reported that for

mouse embryo culture Vero cells secreted small peptides (3-100 kDa) and the authors suggest that these molecules are important to the process of hatching mouse embryos. In our study of bovine embryos, embryotrophic effects of Vero cell secretion were evidenced by the results of a conditioned medium which partly reproduces the effects of somatic Vero cells and similar observations were made by Maeda et al (1996).

Recently, it was demonstrated that the production of bovine blastocysts was also possible in a simple defined medium like SOF, although the concentration of O_2 (5%) is critical since a reduced embryo development at high oxygen concentration is observed (Carolan et al, 1995; Massip et al, 1995). The low oxygen tension is thought to act by reducing auto-oxidative damage to which the embryos are susceptible. Furthermore, improved development was reported when SOF medium is changed every 48 h in order to prevent the accumulation of ammonium as a result of amino acid degradation (Gardner et al, 1994; Trounson et al, 1994). The coculture system always shows itself to be efficacious in producing viable IVF embryos since this system is achieved under routine conditions of 5% CO_2 in air. Furthermore, during coculture with Vero cells, the medium does not need to be renewed until after 7 days of culture. This could be explained by the fact that these cells contribute to embryo development by removing embryotoxic factors like ammonium and also because renewing the medium may deprive embryos of the beneficial autocrine factors produced by the feeder cells (Gardner et al, 1994; Carolan et al, 1995).

The quality of the embryos developed on day 7 on Vero cells was assessed by nuclear counting and was compared to that of blastocysts developed on BOEC. We observed that, after the same period of culture (7 days), blastocysts developed on Vero cells had a significantly higher total number of nuclei than those developed on BOEC ($\overline{X} = 142 \pm 39$ vs $\overline{X} = 88.8 \pm 32.8$). This confirms the belief that the kinetics of embryo development are different according to the cell type support. We noticed that most blastocysts were formed at day 6 on Vero cells compared to day 7 on BOEC and such kinetics are similar to that reported by Galli and Lazzari (1996) for in vivo culture of bovine zygotes in sheep oviducts. Furthermore, the total mean number of cells for day 7 blastocysts developed on Vero cells is similar to that reported by Heyman et al (1995) for in vivo collected day seven blastocysts ($\overline{X} = 161.57 \pm 8.38$). The method that we used for cell number evaluation is more precise than in toto preparation and counting, as the exact total number of nuclei was assessed in serial optical sections of each embryo using a confocal microscope. However, propidium iodide staining did not allow the ratio between inner cell mass and trophoectoderm cells to be evaluated.

The pregnancy rate (58%) observed after single transfer of blastocysts developed on Vero cells confirms the high viability of such blastocysts. Although no strict comparison was made for in vivo survival of in vitro produced blastocysts developed on Vero or BOEC cells in the present work, we believe that the pregnancy rate achieved is at least similar to or higher than previous reports in our laboratory. We obtained 38% pregnancy rate (10/26) after single transfer of in vitro derived blastocysts developed on BOEC (Revel et al 1995).

In conclusion, this study shows that Vero cells, an established line of African monkey kidney cells are a sanitary safe support to produce viable in vitro bovine embryos with limited variations between experiments. This coculture system can be successfully used for developing bovine embryos after other related technologies such as nuclear transfer or gene injection.

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