

The development potential of bovine embryo co-culture with Vero and Vero/BRL cells*

**A.M. Duszewska¹, J. Wojdan², W. Gawron², E. Wenta-Muchalska¹,
B. Waś¹, A. Wiśniewska¹ and Z. Reklewski²**

Institute of Genetics and Animal Breeding, Polish Academy of Sciences,

¹Department of Experimental Embryology,

*²Department of Cattle Genetics and Breeding
Jastrzębiec, 05-552 Wólka Kosowska, Poland*

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ABSTRACT

The objective of this study was to evaluate the effect of two bovine embryo co-culture systems on calving rates. The embryos were co-cultured with Vero or Vero/BRL cells (mixed co-culture) until the blastocyst stage. A higher percentage of blastocysts was observed on Vero/BRL cells, 42.85%, compared with 30.41% on Vero cells ($P \leq 0.05$). The blastocysts from Vero/BRL and Vero cells were transferred to recipients. A higher rate of calving was obtained after transfer of embryos co-cultured with Vero cells (37.50%), compared with Vero/BRL cells (13.16%) ($P \leq 0.01$). A greater loss of pregnancy after transfer of embryos co-cultured with Vero/BRL cells was observed between 35 and 65 days. All calves were born naturally, healthy, and with normal weight. Vero cells better support cattle prenatal development than Vero/BRL cells. Probably Vero/BRL cells lead to epigenetic modifications that are responsible for early foetal resorption.

KEY WORDS: Vero, BRL, embryos, co-culture, cattle

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¹ Corresponding author: e-mail: duszewskaanna@hotmail.com

INTRODUCTION

In vitro production of bovine embryos has applications both in cattle breeding and in biotechnology for cloning, transgenics and creation of chimeras (Gordon, 2003). There are three frequently used systems for culture of bovine embryos from zygote to blastocyst. The most common system relies on sequential culture in several different media: SOFaa (Holm et al., 1999), KSOM (Liu and Foote, 1995), CR1 (Rosenkranz and First, 1994) and CZB (Rehman et al., 1994). Another system employs co-culture with different kinds of cells such as Vero cells (Pegoraro et al., 1998), BRL cells (Reed et al., 1994) or a combination of both (so-called “mixed co-culture”) (Duszewska et al., 2000). A third system uses medium pre-conditioned by the above cells (Maed et al., 1996), but this system is rarely used. All of these systems have similar advantages and disadvantages (Gordon, 2003, review: Orsi and Reischl, 2007). Although they are well-established, there is a need to determine the effect of each system on the developmental potential of embryos by transfer to recipients (Hansen, 2006).

In this study, two co-culture systems were compared to determine the developmental potential of bovine embryos after transfer to recipients. The first system was based on co-culture of embryos with Vero cells and the second co-culture with both Vero and BRL cells (mixed co-culture).

Vero is a kidney epithelial cell line from the green monkey (*Cercopithecus aetiops*). The cells are derived from the kidney, which shares a common mesodermal origin with the genital tract. BRL cells are rat liver epithelial and parenchyma-like cells (*Rattus norvegicus*), which are of endodermal origin.

Bovine embryos have been successfully co-cultured with Vero cells, but the percentage reaching the blastocyst stage is lower than that of embryos co-cultured with BRL cells (Duszewska et al., 2000). According to some authors, the pregnancy rate after transfer of embryos developing on Vero cells is high (Menck et al., 1997). Hence, Vero and BRL cells were combined in an effort to enrich the environment for embryonic development and also to improve prenatal development.

MATERIAL AND METHODS

In vitro maturation of bovine oocytes

Bovine ovaries were obtained from a slaughterhouse and transported within 2 h to the laboratory in phosphate-buffered solution (PBS) with 100 IU/ml streptomycin and 100 IU/ml penicillin at approximately 30°C. Cumulus-oocyte complexes (COCs) were collected by aspiration from follicles (2 to 6 mm in diameter) using a syringe with an 18-gauge needle and an air pump system. COCs

were washed 3 times in TCM199 buffered with 25 mM Hepes supplemented with 10% FBS, 50 µg/ml gentamicin sulphate, 100 IU penicillin and 50 µg/ml streptomycin, and adjusted to pH 7.4. The COCs were matured in TCM199 buffered with 25 mM Hepes supplemented with 10% FBS, 0.02 IU/ml pFSH, 1 µg/ml 17β-oestradiol, 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulphate. A group of COCs (20 immature oocytes) was placed in one well of a 4-well dish and matured in 450 µl TCM199 buffered with 25 mM Hepes and supplemented with 10% FBS, 0.02 IU/ml pFSH, 1 µg/ml 17β-oestradiol, 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulphate. The COCs were matured for 24 h at 38.5°C in 5% CO₂ in humidified air (Marguant-Le Guienne et al., 1989).

In vitro fertilization of bovine oocytes

Frozen spermatozoa obtained from a single bull were thawed in a waterbath (37°C), centrifuged (200xG) for 10 min, and resuspended in 2 ml Sp-TALP medium containing 6 mg/ml BSA fraction V adjusted to pH 7.4. Spermatozoa were prepared by the "swim-up procedure" (Parrish et al., 1986). After maturation, COCs were washed 3 times in fertilization medium Fert-TALP supplemented with 6 mg/ml BSA FAF, 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulphate. Groups of matured oocytes (10 COCs) were placed in 4-well dishes in 450 µl Fert-TALP supplemented with 6 mg/ml BSA FAF, 0.2 mM Na pyruvate, 50 µg/ml gentamicin sulphate, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 2 µg/ml heparin. Spermatozoa were used at a final concentration of 1×10⁶/ml. The COCs and spermatozoa were co-cultured for 18 h at 38.5°C in 5% CO₂ in humidified air (Marguant-Le Guienne et al., 1989).

In vitro culture of bovine embryos

At 20 h post-insemination, the cumulus cells were removed and the zygotes washed in Menezo B2 medium (ART of CCD) supplemented with 10% FBS (GIBCO) and placed in 40-µl drops of Menezo B2 medium supplemented with 10% FBS under mineral oil. The zygotes were randomly allocated to one of the experimental groups: Vero and Vero/BRL.

Embryos were co-cultured until day 7, i.e. 168 h post insemination, at 38.5°C in 5% CO₂ in humidified air. During culture the medium was renewed twice (at 48 h, 20 µl of medium was removed and 20 µl of Menezo B2 supplemented with 10% FBS was added; and at 144 h, 20 µl of medium was removed and 20 µl of Menezo B2 without serum was added).

Culture of Vero and BRL cells

Frozen samples of established Vero and BRL cell lines (ATCC, Maryland, USA) were used to prepare respective Vero cell monolayers and BRL cell monolayers. Cell lines were thawed and cells seeded at a concentration of $1 \times 10^6/\text{ml}$ in 25 cm^2 culture flasks in the TCM 199 medium supplemented with 10% FBS, $50 \mu\text{g}/\text{ml}$ gentamicin sulphate, 100 IU penicillin, and $50 \mu\text{g}/\text{ml}$ streptomycin and cultured at 38.5°C in 5% CO_2 in humidified air. The medium from both the Vero and BRL cultures was removed and replaced with fresh medium after 2 days. BRL cells (after 4 days of culture) and Vero cells (after 7 days of culture) were trypsinized (0.25% trypsin) for passages to prepare the monolayers and microdrops for culture of embryos.

Preparation of Vero cells in drops under mineral oil

Vero cells were placed at a concentration of $2 \times 10^3/10 \mu\text{ml}$ into a microdrop (40 μml) of Menezo B2 (ART of CCD) supplemented with 10% FBS under mineral oil and cultured at 38.5°C in 5% CO_2 in humidified air. The next day, the zygotes were placed in a microdrop.

Preparation of Vero/BRL cells in drops under mineral oil

Vero cells were first placed at a concentration of $1 \times 10^3/10 \mu\text{ml}$ into a microdrop (40 μml) of Menezo B2 (ART of CCD) supplemented with 10% FBS under mineral oil. After 30 min, BRL cells were added at the same concentration to the same drop, which was then cultured at 38.5°C in 5% CO_2 in humidified air. One day later, the zygotes were placed in the microdrop.

Embryo transfer and offspring evaluation

Oestrus synchronization of the recipients was induced by injecting 2 ml (0.5 mg) of the prostaglandin $\text{F}_{2\alpha}$ -analogue, Cloprostenol (Bioestrovvet-Vetoquinol, Gorzów, Poland) every 11 days. Seven to eight days after the standing heat, one fresh embryo was transferred to the uterine horn ipsilateral to the ovary displaying a *corpus luteum*. Embryos were transferred to recipients in Embryo Transfer Medium (BioLife Transfer Medium, Agtech Inc., USA). Recipients were monitored daily for heat behaviour and examined by ultrasound after 5 weeks and then monthly to confirm pregnancy. After calving, offspring were weighed and examined by veterinary doctors.

Statistical analysis

Rates of cleavage and blastocyst formation per zygote and rate of development after transfer to recipients were analysed by *Chi-square* tests. Birth weight is given

as the mean value \pm standard deviation. Probabilities (P-values) of less than 0.05 were regarded as statistically significant and less than 0.01 as highly statistically significant.

RESULTS

The development of zygotes co-cultured with Vero or Vero/BRL cells was compared on day 2 (48 h post insemination) and on day 7 (168 h post insemination) (Table 1). On day 2 no significant differences were observed in embryo development (cleaved embryos) between the two co-culture systems.

Table 1. *In vitro* development of bovine embryos co-cultured with Vero and Vero/BRL cells

Culture system	No. of zygotes	Cleaved embryos %	Blastocysts %
Vero	240	198 (82.5)	73 (30.41) ^a
Vero/BRL	238	201 (84.45)	102 (42.85) ^b

^{ab} statistical difference ($P \leq 0.05$)

On day 7, a higher percentage of successfully developed blastocysts was observed on mixed co-culture - Vero/BRL cells (42.85%), whereas a lower percentage of blastocysts was observed on Vero cells (30.41%). The difference between Vero and Vero/BRL was statistically significant ($P \leq 0.05$).

The results of embryo transfer are presented in Table 2. On day 35, no significant difference was observed in prenatal development of embryos between Vero and Vero/BRL cells. On day 65, a higher percentage of prenatal

Table 2. The results of embryo transfer to recipients

Culture system	No. of transferred blastocysts	No. of pregnancies on day 35, %	No. of pregnancies on day 65, %	Calving rate of offspring	Birth weight $x \pm$ SD
Vero	72	31 (43.05)	27 (37.50) ^a	27 (37.50) ^c	32.6 \pm 3.87
Vero/BRL	76	34 (44.74)	12 (15.78) ^b	10 (13.16) ^d	34.2 \pm 4.17

^{a,b,c,d} indicate values showing a statistically significant difference between culture systems (^{ab} $P \leq 0.05$, ^{cd} $P \leq 0.01$)

development was found on Vero cells (37.50%), whereas a lower percentage, on Vero/BRL cells (15.78%). The difference between the two co-culture systems was statistically significant ($P \leq 0.05$). A higher calving rate was observed from embryos

co-cultured with Vero cells (37.50%) as compared with Vero/BRL cells (13.16%). The difference between groups was highly statistically significant ($P \leq 0.01$). All calves were born naturally and healthy. Independent of culture system, the birth weight of all calves was similar.

DISCUSSION

Bovine embryos developed more efficiently to the blastocyst stage on Vero/BRL cells: 42.85% (mixed co-culture) than on Vero cells: 30.41% (Table 1). This supports our previous results, in which the difference between the two co-culture systems was similar: 40.50% on Vero/BRL cells vs 27.25% on Vero cells (Duszewska et al., 2000). Also in other studies, a lower percentage of blastocysts was obtained from co-culture of embryos with both Vero cells, about 30% (Menck et al., 1997; Pegoraro et al., 1998) and BRL cells, about 35% (Farin et al., 1995; Hernandez-Ledezma et al., 1996).

Somatic cells may support embryo development through two possible mechanisms. First, somatic cells could remove deleterious components from the culture medium, protect against agents of oxidative stress and/or modulate the physico-chemical conditions of the medium. Some authors suggest that Vero cells may specifically assist by metabolizing or absorbing inhibitory substances from the culture medium. Second, somatic cells may also secrete embryotrophic factors (review: Orsi and Reischl, 2007). Both Vero and BRL cells secrete many such factors, including colony stimulating factor (CSF), epidermal growth factors (EGFs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), insulin growth factors (IGF-I and -II), interleukins (ILs), leukaemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), stem cell factor (SCF) and transforming growth factors ($TGF-\alpha$, $-\beta_1$ and $-\beta_2$) (Duszewska et al., 2000).

The aim of this research was to compare the developmental potential of embryos co-cultured with Vero and Vero/BRL cells after transfer to recipients. Generally, both embryo yield and embryo quality contribute to embryo transfer efficiency (Rizos 2002). *In vitro* conditions during maturation of oocytes, fertilization and embryo development can lead to a short-term response and long-term consequences for prenatal and postnatal development. Altered intracellular signalling, metabolic stress, changes in gene expression, apoptosis, cell proliferation, chromosomal abnormality and epigenetic modifications may all have short-term effects. By contrast, long-term consequences of *in vitro* culture can include reduced implantation capacity, unbalanced foetal/placental allocation, altered maternal nutrient provision and abnormal foetal growth rate (Loneragan et al., 2006). In farm animals, these long-term consequences lead to abnormal birth weight and postnatal growth abnormality, referred to as Large Offspring Syndrome (LOS) (van Wageningen et al., 2000; Bertolini et al., 2002). Sometimes, but not

usually, LOS has been associated with *in vitro* production of embryos, including nuclear transfer and pronuclear injection (Young and Fairburn 2000; review: van Wagtgendonk et al., 2000; Renard et al., 2002; Duszewska et al., 2003, 2004).

In our experiment more offspring were obtained after transfer of embryos co-cultured with Vero cells (37.5%) than with Vero/BRL (13.16%) (Table 2). Generally, the calving rate after transfer of cultured embryos to recipients is lower (about 30-40 %) than of embryos obtained *in vivo* (about 70%) (Pomar et al., 2005). However a calving rate of 13.16% after transfer of embryos co-cultured with Vero/BRL cells is drastically lower. The high pregnancy loss occurred between days 35 and 65.

However, embryos developed better to the blastocyst stage with Vero/BRL cells than with Vero cells, but after transfer, embryos co-cultured with Vero cells developed better. The difference between co-culture systems could be explained by excessive concentrations of some growth factors and proteins in the mixed co-culture system (Vero/BRL). These results conflict with the opinion that mixed co-culture is more effective than systems using a single cell line (Orsi and Reischl, 2007).

Probably, Vero/BRL cells lead to changes in epigenetic modifications during development. Epigenetic modifications of DNA and chromatin are important for genome function during prenatal and postnatal development. Environmental factors during *in vitro* development, as well as oocyte maturation and possibly fertilization, can alter epigenetic modifications and thus have short- and long-term effects on development (Reik et al., 2003).

CONCLUSIONS

Vero cells better support bovine prenatal development than Vero/BRL cells. It is likely that Vero/BRL cells led to epigenetic modifications that manifested later in development and were responsible for early foetal resorption.

REFERENCES

- Bertolini M., Mason J.B., Beam S.W., Carneiro G.F., Sween M.L., Kominek D.J., Moyer A.L., Famula T.R., Sainz R.D., Anderson G.B., 2002. Morphology and morphometry of *in vivo*- and *in vitro*-produced bovine conception from early pregnancy to term and association with high birth weights. *Theriogenology* 58, 973-994
- Duszewska A.M., Reklewski Z., Pieńkowski M., Karasiewicz J., Modliński J.A., 2000. Development of bovine embryos on Vero/BRL cell monolayers (mixed co-culture). *Theriogenology* 54, 1239-1247
- Duszewska A.M., Rosochacki S.J., Kozikova L., Cybulska M., Korwin-Kossakowski M., Waś B., Połozynowicz J., Wicińska K., Szydlik H., 2003. The use of GFP to select bovine embryos. *J. Anim. Feed Sci.* 12, 71-81

- Duszevska A.M., Wojdan J., Gawron W., Lipiński D., Piliszek A., Wenta-Muchalska E., Waś B., Słomski R., Reklewski Z., 2004. Obtaining calves after transfer of the gene construct microinjected embryos (in Polish). *Med. wet.* 60, 1323-1325
- Farin C.E., Hasler J.F., Martus N.S., 1995. Comparison of Menezzo B2 and TCM-199 media for *in vitro* production of bovine blastocysts. *Theriogenology* 43, 210 (Abstr.)
- Gordon I., 2003. Laboratory production of cattle embryos. In: G.J. Persley (Editor). *Biotechnology in Agriculture Series, No. 27*. 2nd Edition. CABI Publishing
- Hansen P., 2006. Realizing the promise of IVF in cattle - an overview. *Theriogenology* 65, 119-125
- Hernandez-Ledezma J.J., Villanueva C., Sikes J.D., Kubisch H.M., 1996. Increasing the rate of blastocyst formation and hatching from *in vitro* produced bovine zygotes. *Theriogenology* 46, 961-969
- Holm P., Booth P.J., Schmidt M.H., Greve T., 1999. High bovine blastocysts development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* 52, 683-700
- Liu Z., Foote R. H., 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent CO₂. *Biol. Reprod.* 53, 786-790
- Lonergan P., Fair T., Corcoran D., Evans A.C.O., 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology* 65, 137-152
- Maed A.J., Kotsuji F., Negami A., Kamitani N., Tominaga T., 1996. *In vitro* development of bovine embryos in conditioned media from bovine granulosa cells and Vero cells cultured in exogenous protein- and amino acid-free chemically defined. Human Tubal Fluid medium. *Biol. Reprod.* 54, 930-936
- Marguant-Le Guienne B., Gerard M., Solari A., Thibault C., 1989. *In vitro* culture of bovine eggs fertilised either *in vivo* or *in vitro*. *Reprod. Nutr. Develop.* 29, 559-568
- Menck M.C., Guyader-Joly C., Peynot N., Le Bourhis D., Lobo R.B., Renard J.P., Heyman Y., 1997. Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems. *Reprod. Nutr. Develop.* 37, 141-150
- Orsi N.M., Reischl J.B., 2007. Mammalian embryos co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* 67, 441-458
- Parrish J.J., Susko-Parrish J.L., Leibfried-Rutledge M.L., Critse E.S., Eyestone W.H., First N.L., 1986. Bovine *in vitro* fertilisation with frozen-thawed semen. *Theriogenology* 25, 591-600
- Pegoraro L.M.C., Thuard J.M., Delalleau N., Guerin B., Deschamps J.C., Marquant-Le Guienne B., Humblot P., 1998. Comparison of sex ratio and cell number of IVM-IVF bovine blastocysts co-cultured with bovine oviduct epithelial cells or with Vero cells. *Theriogenology* 49, 1579-1590
- Pomar F.J., Teerds K. J., Kidson A., Colenbrander B., Tharasanit T., Aguilar B., Roelen B.A.J., 2005. Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. *Theriogenology* 63, 2254-2268
- Reed W.A., Suh T.K., Bunch T.D., White K.L., 1996. Culture of *in vitro* fertilized bovine embryos with bovine epithelial cells, Buffalo Rat Liver (BRL) cells or BRL-cell-conditioned medium. *Theriogenology* 45, 439-449
- Rehman N., Collins A.R., Suh T.K., Wright R.W., 1994. Development of *in vitro* matured and fertilized bovine oocytes co-culture with Buffalo Rat Liver cells. *Theriogenology* 41, 1453-1462
- Reik W., Santos F., Dean W., 2003. Mammalian epigenetics: reprogramming the genome for development and therapy. *Theriogenology* 59, 21-32
- Renard J.P., Zhou Q., Lebourhis D., Chavatte-Palmer P., Hue I., Heyman Y., Vignon X., 2002. Nuclear transfer technologies: between successes and doubts. *Theriogenology* 57, 203-222
- Rizos D., Ward F., Duffy P., Boland M.P., Lonergan P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: Implication for blastocysts yield and blastocysts quality. *Mol. Reprod. Dev.* 61, 234-248

- Rosenkranz Jr. C.F. , First N.L., 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. *J. Anim. Sci.* 72, 434-437
- Van Wagendonk-de Leeuw A.M., Mullaart E., de Roos A.P. W., Merton J.S., Den Daas J.H.G., Kemp B., De Ruigh L., 2000. Effects of different reproduction techniques: AI, MOET or IVP on health and welfare of bovine offspring. *Theriogenology* 53, 575-597
- Young L.E., Fairburn H.R., 2000. Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology* 53, 627-648