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Developmental competence of bovine IVM/IVF oocytes under different co-culture conditions

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ABSTRACT

The experiment was carried out to compare the influence of different culture systems on the developmental potential of bovine embryos derived from IVM/IVF oocytes. The investigations included comparison of the developmental competence of IVM/IVF zygotes derived from oocytes cultured in large (>30 oocytes/well) and small (1-5 oocytes/drop of medium) groups and co-cultured with: Buffalo rat liver cells (BRL), African Green monkey kidney (VERO) and bovine oviduct epithelial cells (BOEC).

The highest number of embryos derived from IVM/IVF oocytes maturing in large groups developed to blastocysts in co-culture with VERO (45.9%), followed by BRL (30.9%) and BOE (36.1%) cells. The hatching ability of these blastocysts did not differ significantly for BRL and VERO co-cultures (respectively 74.5 and 58.0%) and was significantly lower for those co-cultured with BOEC (34.7%). In contrast to the oocytes maturing in large groups, the developmental competence of oocytes of the small group cultures was independent of the feeder layer used for co-culture and 19.4 to 27.1% of these oocytes reached the blastocyst stage. No significant differences were noticed in either the blastocyst rates or in the hatching ability of these blastocysts developing in co-culture with BRL, VERO and BOE cells. The mean number of cells in the blastocyst of the large group culture developing in co-culture with BRL cells on day 8 was 112.0±27.7 while the blastocysts developing in co-cultures with VERO and BOE cells contained 76.8±20.0 and 94.0±23.6 cells, respectively.

KEY WORDS: cattle, IVM/IVF, co-culture, BRL, VERO, BOEC

INTRODUCTION

In recent years, oocyte *in vitro* maturation and fertilization in cattle has been greatly improved. Many systems for culture of early-stage bovine embryos deri-

ving from IVM/IVF oocytes have been used. A culture system after IVF is crucial to embryo production technology. It is evident that embryos require a well-balanced microenvironment to develop properly. There are several successful embryo culture systems that allow fertilized zygotes to develop to post-compaction stages *in vitro*. Culture systems can essentially be divided into two groups: a. those in which somatic cells are included in a co-culture system or at least the medium is exposed to somatic cells (conditioning), b. those which have no dependence on somatic cells.

By far co-culture with somatic cells is the much preferred system for development of bovine embryos. Various types of cells can be used as feeder layer to improve the rate and the quality of IVP embryos, i.e. trophoblastic vesicles (Camous et al., 1984; Heyman et al., 1987; Aoyagi et al., 1990), granulosa cells (Goto et al., 1988; Mochizuki et al., 1991; Behboodi et al., 1992), uterine endometrial cells (Kuzan and Wright, 1982; Marquant-Le Guienne et al., 1989), bovine oviduct epithelial cells "BOEC" (Gandolfi and Moor, 1987; Aoyagi et al., 1990; Behboodi et al., 1992; Kątska et al., 1995), Buffalo rat liver "BRL" cells (Voelkel and Hu, 1992; Hawk and Wall, 1994; Bevers, 1995) and African Green monkey kidney "VERO" cells (Guyader-Joly et al., 1996; Pegoraro et al., 1996). Bovine embryos derived from IVM/IVF procedures are most frequently co-cultured with BOEC to achieve development to the blastocyst stage. The oviducts for BOEC recovery commonly come from slaughterhouse cattle and, due to heterogeneity in cell origin, they can affect co-culture conditions. We have proven that cryoconserved BOEC can support embryo development in a way similar to that of co-culture with fresh cells (Kątska et al., 1995). Recently, well-defined, established lines of Buffalo rat liver cells (BRL) and/or African Green monkey kidney cells (VERO) have been successfully used for supporting embryo development. The use of BRL or VERO cell lines, easy to handle (passages) and freeze, offers the advantages of working with identifiable cells for the co-culture system (Voelkel and Hu, 1992; Hawk and Wall, 1994; Bevers, 1995; Guyader-Joly et al., 1996; Pegoraro et al., 1996).

The aim of the study was to compare the developmental competence of IVM/IVF bovine zygotes deriving from oocytes cultured in large and small groups and then co-cultured with: Buffalo rat liver cells (BRL), African Green monkey kidney cells (VERO) and bovine oviduct epithelial cells (BOEC).

MATERIAL AND METHODS

Oocyte recovery and in vitro maturation

Ovaries of heifers and cows were collected at the local abattoir and were transported to the laboratory at 28 to 30°C within 2 to 3 h of slaughter. The ovaries were

washed 3 times in warm PBS supplemented with kanamycin[1] (0.075 g/l). Cumulus-oocyte complexes were freed from ovaries following the isolation and subsequent rupture of vesicular follicles >2 mm in diameter in TCM 199 manipulation medium, Earle's salt with HEPES 25 mM[1] containing 10% fetal calf serum (FCS)[1]. Oocytes with compact and dense cumulus oophorus and evenly granulated cytoplasm were cultured in TCM 199[1] (Earle's salt, pH 7.4) supplemented with 20% oestrous cow serum (ECS)[2] and 25 mM $NaHCO_3$[1] and an additional 3 to 5 x 10^6 granulosa cells/ml. Granulosa cells were collected from healthy follicles 3 to 5 mm in diameter (Kątska et al., 1995). The volume of medium used for maturation was related to the number of oocytes per group and ranged from 10 ml to 2 ml. Oocytes were cultured for 23 to 24 h at 39°C under 5% CO_2 in air at maximum humidity.

Sperm preparation and in vitro fertilization

Motile spermatozoa were obtained by discontinuous density gradient centrifugation (90%:45% Percoll[3]) of frozen-thawed spermatozoa (Lazzari and Galli, 1994). Briefly, spermatozoa were layered on the top of a 45% Percoll solution in TALP-capacitation medium (Parrish et al., 1988) in a centrifuge tube. The sample was centrifuged for 30 min at 500 x g and the supernatant was removed. The sperm pellet was washed once with TALP-capacitation medium (500 g for 10 min) and resuspended (1.5-2 x 10^6 sperm/ml) in 40 ml drops of TALP-IVF medium (containing 10 µg/ml heparin[1] and mixture of penicillamine[1], hypotaurine[1] and epinephrine[1]) under mineral oil[1] (Kątska et al., 1996). Gametes were incubated together for 20 to 22 h at 39°C under 5% CO_2 in air.

Zygotes co-culture to the blastocyst stage

After 20 to 22 h of fertilization, the oocytes were washed 3 times with TALP washing medium (Parrish et al., 1988) to remove corona cells and attached spermatozoa, then transferred into 40 ml drops of Menezo B_2[4] medium under mineral oil, and cultured approximately 20 to 24 h (40 to 46 h post insemination). Then, the uncleaved ova were discarded and embryos were randomly distributed into the treatment groups (co-culture with BRL, VERO or BOE cells). Co-culture was carried out up to the time the embryos reached the blastocyst/hatched blastocyst stage. For all co-cultures Menezo B_2 medium supplemented with 10% of FCS was used. Embryo development was recorded at intervals of 48 h up to the time corres-

[1] Sigma Chemical Company, St. Louis, Mo, USA
[2] Serum prepared in our laboratory
[3] Pharmacia Biotech AB, Uppsala, Sweden
[4] bioMerieux sa, Marcy/Etoile, France

ponding to 8 d after insemination (i.e., to the blastocyst stage) and simultaneously the medium was partially (1/3) exchanged. The culture of some blastocysts was extended up to 10 d after insemination i.e., to the hatched blastocyst stage.

Preparation of somatic cells

The BRLC (BRL 3A) and VERO cultures were obtained frozen from ECACC, Salisbury, UK. Cell samples were thawed in a 37°C water bath, washed by centrifugation in 10 ml of TCM 199 manipulation medium and suspended in TCM 199 culture medium containing 10% FCS, 1% penicillin G[1]/streptomycin sulphate[1] (10000 UI/10000 μg/ml), and 1% fungizone[5]. Cells were seeded at the concentration: a. 1 x 10^6 cells/10 ml medium per flask (for passages); b. 1 x 10^4 cells per ml medium per well (for co-culture with a group of >30 zygotes); c. 1 x 10^3/40 μl medium per drop (for co-culture with the small group of zygotes). At confluency, the wells and drops were ready for co-culture. The medium for co-culture i.e., Menezo B_2 + 10% FCS, had been exchanged before placing of zygotes.

The BOEC were obtained at the luteal phase of the cycle from slaughtered heifers at a local abattoir. Epithelial cells stripped from the oviducts were washed twice in TCM 199 manipulation medium, and then the cells were suspended in culture medium at a concentration 2 to 3 x 10^6 cells/ml. Then 0.5 ml of the suspension was placed in a 4-well multidish previously coated with collagen gel (for co-culture with a large group of zygotes) or 40 ml per drop of the suspension were placed under mineral oil in a plastic Petri dish (for co-culture with a small group of zygotes). The BOEC was prepared 2 or 3 days before the start of co-culture with zygotes. Before placing zygotes the medium was changed, and then exchanged every 48 h (Kątska et al., 1995).

Blastocyst assessment

Some blastocysts of the large group cultures recovered on day 8 and 10 of co-culture of zygotes with BRL, VERO and BOE cells were evaluated by fluorescent microscopy after the blastocysts had been incubated in a medium containing 5 μg/ml Hoechst 33342 for 10 min. The number of nuclei in each blastocyst was counted twice, and the mean number was recorded.

Experimental design

The experiment was conducted to compare the influence of different culture systems on the developmental potential of co-cultured bovine embryos. The ob-

[5] GIBCO, BRL, Life Technologies

jective of the experiment was to investigate the effects of somatic cells used for co-culture [Buffalo rat liver cells (BRL), African Green monkey kidney (VERO) and bovine oviduct epithelial cells (BOEC)] on the developmental capacity of bovine IVM/IVF oocytes that were matured and cultured in the large (>30 oocytes/well) or in the small (1-5 oocytes/drop) groups. The volume of the medium used for co-culture with group of ≥30 oocytes was 500 µl, while co-culture of small groups of oocytes was performed in 40 µl per drop under mineral oil. The quality of the recovered blastocysts was evaluated both by their hatching ability and on the basis of the number of cells in the blastocysts recovered on day 8 and 10.

Statistical analysis

Cleavage was examined at 42 to 46 h post insemination and represented that portion of oocytes completing at least first mitotic division. Frequency of blastocyst development was calculated from the total number of oocytes used for fertilization, and hatched blastocysts from the number of blastocysts which were left in culture for up to 10 days. Differences among treatment means were tested by the Chi-square test.

RESULTS

Comparison between different sources of somatic cells used as the feeder layer has shown statistically significant differences in the proportion of zygotes derived from oocytes maturing in the large groups (≥30 oocytes) that developed to the blastocyst stage. The highest number of embryos developed into blastocysts when co-cultured with VERO (45.9%), as compared with BRL (30.9%) or BOE (36.1%) cells. The hatching ability of blastocysts derived from large group co-cultures with BRL and VERO cells amounted to 74.5 and 58.0%, respectively, and was significantly lower for those co-cultured with BOEC (34.7%; Table 1).

In contrast to the oocytes maturing in the large groups, the cleavage ability of oocytes of the small group cultures was significantly reduced (495/603, 82.1% vs 254/383, 66.3%; $P<0.001$). The developmental competence of small group cultures was independent of the feeder layer used for co-culture and 19.4 to 27.1% of these oocytes reached the blastocyst stage. No statistical differences were noticed in the blastocyst rates and the hatching ability of these blastocysts developing in co-culture with BRL, VERO and BOE cells (Table 1).

Comparison between the developmental ability of oocytes maturing in the large and small groups has shown a significant difference ($P<0.001$) in the blastocyst rates (45.9% vs 19.4%) only for co-cultures with VERO cells. For the other co-culture systems i.e., with BRL or BOE cells, only a tendency of the small group oocytes to lower developmental competence was noticed (Table 1).

TABLE 1

In vitro development of bovine zygotes co-cultured with somatic cells: oocyte maturation and embryo co-culture were carried out in the large groups (>30 oocytes/well) or in the small groups (1-5 oocytes/drop of medium)

Co-culture system	Group[a]	No of oocytes/ no of repl	No (%)[b] of cleaved embryos	No (%)[a] of obtained blastocysts	No (%)[c] of hatching
BRL cells	L	152/5	118 (77.6)	47 (30.9) **V	35 (74.5) ***O
	l	126/3	90 (71.4)	30 (23.8)	17 (56.7)
VERO cells	V	135/4	112 (83.0)***v	62 (45.9) *O; **L; ***v	36 (58.0) *O
	v	124/3	80 (64.5)***V	24 (19.4)***V	12 (50.0)
BOE cells	O	316/9	265 (83.9)***o	114 (36.1) *V	40 (34.7) *V; ***L
	o	133/3	84 (63.2)***O	36 (27.1)	14 (38.9)

[a] groups labelled with the capital letters (L,V,O) consisted of >30 oocytes/well, and with small letters (l,v,o) consisted of 1-5 oocytes/drop of medium

[b] percentages were calculated in relation to the number of oocytes used for IVM/IVF

[c] percentages were calculated in relation to the total number of recovered blastocysts

c^2 test: * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Comparing the number of cells in the blastocysts originating from the large group co-cultures with different sources of somatic cells we can conclude that the co-culture with BRL seems to create more appropriate conditions for normal development of bovine blastocysts. The mean number of cells in the blastocyst recovered from co-culture with BRL cells at day 8 was 112±27.7 while the blastocysts developing in co-cultures with VERO and BOE cells contained 76.8±20.0 and 94.0±23.6 cells, respectively (Table 2).

TABLE 2

Number of blastomeres in bovine blastocyst developed in the large group co-culture with BRL, VERO or BOE cells

Co-culture conditions	No of blastocysts used	Number of cells in the blastocyst recovered at:	
		day 8 (ranging)	day 10 (ranging)
BRL cells	19	112.0±27.7 (78 – 172)	149.0 ±53.4 (110 – 240)
VERO cells	31	76.8±20.0 (40 – 116)	152.4±35.3 (113 – 198)
BOE cells	43	94.0±23.6 (71 – 134)	112.3±34.7 (61 – 194)

DISCUSSION

Although several experiments on bovine *in vitro* embryo production have been carried out, the results have been subject to considerable interlaboratory and replicate variation. It is well known that the influence of the bull and, particularly, interbull variation in fertilizability *in vitro* plays a key role in embryo production (Eyestone and First, 1989; Hillery et al., 1990; Barandi et al., 1993; Kątska et al., 1996). Moreover, recommended procedures for particular steps of embryo production *in vitro* (i.e., oocyte maturation, fertilization and embryo culture) differ considerably among laboratories, so direct comparison of the embryo yield between different culture systems is difficult.

In our earlier experiments (Kątska et al., 1995), we proved that BOEC can be collected regardless of the phase of the donor cycle (including that of superovulated animals), since BOEC similarly supported developmental ability and blastocyst formation of IVM/IVF-derived bovine zygotes. We also demonstrated that cryopreservation does not affect the properties of oviduct epithelial cells which could provide embryonic growth stimulatory components and/or remove embryotoxic substances from the culture medium in a way similar to that of fresh, nonpreserved cells. This advantage creates the opportunity to use several sources of cryopreserved somatic cells (BRL, VERO, BOEC) in a system for the *in vitro* coculture of IVM/IVF-derived bovine zygotes, which would be especially useful in large-scale production of embryos for commercial purposes. This is important because the use of cryopreserved cells in a co-culture system may obviate concerns about the potential of living homologous tissue as a source of infection/disease, since cryopreserved cell stocks could be screened prior to use.

This study clearly indicates that a number of factors, i.e., the source of somatic cells and the number of embryos cultured together in the same drop or well seem to play an important role in regulating the development of bovine IVM/IVF oocytes to the blastocyst stage. The results of our experiment showed that higher rates of blastocyst development and hatching were obtained when zygotes, produced from IVM/IVF oocytes developing in the large group, were co-cultured with BRL or VERO cells compared to those co-cultured with BOE cells.

As has been assessed by other authors, 7- to 8-day-old bovine embryos developed *in vivo* (recovered from superovulated cattle) contain about 115-160 cells in the blastocyst stage (Skrzyszowska and Smorąg, 1989; Heyman et al., 1995; Van Soom et al., 1997). These numbers were only slightly lower for the *in vitro* produced blastocysts developing in co-culture with BRL, therefore there is no reason to believe that the cells' number can be the sole indicator of their quality. Moreover, the asynchrony of blastocyst development must be noticed regardless of the co-culture system used. Therefore, after 10 days of culture, embryos from early blastocyst up to hatched blastocyst stages were observed. Basing a comparison of

the quality of blastocysts developed in the different co-culture systems (with BRL, VERO and BOE cells) on the basis of cell number in 8- and 10-day-old embryos and the ability of blastocysts to hatch indicates that co-culture with BRL seems to create more optimal conditions in comparison to the other systems. Moreover, it has been shown by Voelkel and Hu (1992) that co-culture on BRL cells for at least 24 h prior to freezing improves the freezing resistance of embryos.

Among other factors influencing the quantity and quality of IVP blastocysts an important role seems to be played by regulation of development conditioned by the number of embryos cultured together in the same drop or well. A trend towards increasing blastocyst cell numbers was found when sheep zygotes were cultured in increasing group sizes (Gardner et al., 1994). In cattle, both under cell-free culture conditions and in co-culture with granulosa cell monolayers, the rate of day 8 blastocyst development and quality was dramatically reduced when embryos were cultured singly compared to large groups (groups: 7.5±1.3% and 37.4±4.9%; singles: 1.1±0.8% and 9.9±2.6%; O'Doherty et al., 1996). Thibodeaux et al. (1995) showed the beneficial effects of incubating bovine embryos in larger groups due to platelet-derived growth factor. Nevertheless, culture of small numbers or single oocytes is desirable for several reasons, such as recovery of embryos from individual donors using transvaginal aspiration, the OPU technique, correlating the relationship between follicular properties and oocyte developmental competence and the influence of nutrition on reproduction. Although the results of our experiment have shown the significant difference between the large and small group co-cultures with VERO cells and a trend towards reducing the developmental competence of the small group oocytes co-cultured with BRL and BOE cells, it was possible to obtain approximately 20% blastocysts, regardless of the co-culture system used. It is to be hoped that closer parity between the large- and the small-group derived embryos should occur as knowledge of the requirements for oocyte and embryo development increases.

CONCLUSIONS

Summarizing the developmental potential of bovine IVM/IVF-derived embryos in different co-culture systems, we conclude that embryos show relatively great tolerance to varying co-culture conditions. Essential factors required for normal embryo development, cell line differentiation and embryo viability are derived from somatic cells in the media, and these probably contribute to providing the necessary culture conditions for embryo development.

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STRESZCZENIE

Rozwój zarodków bydlęcych uzyskanych z dojrzewających i zapładnianych *in vitro* oocytów w zróżnicowanych systemach współhodowli

Porównano wpływ różnych systemów hodowli zarodków uzyskiwanych z dojrzewających i zapładnianych *in vitro* oocytów bydlęcych. Porównywano rozwój zygot uzyskiwanych z oocytów dojrzewających w dużych (>30 oocytów/naczynko) i małych (1-5 oocytów/kroplę pożywki) grupach. Do współhodowli zygot stosowano 3 rodzaje komórek somatycznych: komórki wątroby szczura (Buffalo rat liver cells -BRL), nerki afrykańskiej małpy zielonej (kidney of African Green monkey -VERO) i nabłonka jajowodu bydlęcego (bovine oviduct epithelial cells -BOEC).

Najwyższa liczba zarodków uzyskanych z oocytów dojrzewających w dużych grupach rozwijała się do blastocyst we współhodowli z komórkami VERO (45,9%) w porównaniu z BRL (30,9%) czy BOEC (36,1%). Zdolność do wylęgania się tych blastocyst nie różniła się istotnie we współhodowli z komórkami BRL i VERO (odpowiednio 74,5 i 58,0%), natomiast była istotnie niższa we współhodowli z BOEC (34,7%). W przeciwieństwie do oocytów dojrzewających w dużych grupach, zdolności rozwojowe zarodków uzyskiwanych z oocytów hodowanych w małych grupach nie zależały od rodzaju komórek somatycznych użytych do współhodowli i 19,4 do 27,1% tych oocytów osiągało stadium blastocysty. Nie stwierdzono statystycznie istotnych różnic w odsetku uzyskanych blastocyst i ich zdolności do wylęgania się we współhodowli z komórkami BRL, VERO i BOEC. Średnia liczba komórek blastocysty uzyskanej z oocytów dojrzewających w dużej grupie , a następnie współhodowanych z komórkami BRL w dniu 8 wynosiła 112,0±27,7, podczas gdy blastocysty rozwijające się w obecności komórek VERO i BOEC zawierały odpowiednio 76,8±20,0 i 94,0±23,6 komórek.