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Original article

Three year results of in vitro production of bovine embryos in serum-poor bovine oviduct conditioned medium. An overview

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Summary — This paper presents a synthesis of 3 year results of in vitro production of bovine embryos in medium previously conditioned by bovine oviduct epithelial cells. In Louvain-la-Neuve, Belgium, a total of 18 356 oocytes were matured and inseminated in vitro: 13 967 (76%) had cleaved at 3 days post-insemination and 3 593 (26%) became blastocysts using this culture system. Our data show that conditioned medium can be stored frozen for up to 3 years without significant loss of activity and is resistant to lyophilization. One single batch of conditioned medium was tested within the same period in four different laboratories and yielded variable results: 27 and 37% blastocysts/cleaved embryos in two of them and only 7 and 0% in the two others whereas in each case more than 30% blastocysts were obtained with the local reference co-culture system. In one laboratory, the batch of oil used to overlay the culture drops had a detrimental effect on the blastocyst rate in conditioned medium but not in co-culture.

bovine / embryo development / in vitro culture / conditioned medium / oviduct

Résumé —Développement des embryons bovins dans du milieu sans sérum conditionné par des cellules d'oviducte bovin. Bilan après trois années de culture. *Nous avons développé en 1992 un système de culture de l'embryon bovin dans du milieu 199 sans sérum préalablement condi-*

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tionné par une monocouche de cellules d'oviducte bovin. Ce milieu a été testé comme support de culture dans quatre laboratoires distincts, la stabilité du milieu a été éprouvée, le rendement du système a été évalué par l'utilisation extensive de différents lots de milieux. Un bilan des données récoltées est présenté dans cet article. Nos résultats montrent que ce milieu peut être conservé congelé pendant au moins trois ans sans perte significative de son activité embryotrophe et qu'il résiste à la lyophilisation. Une synthèse des résultats obtenus à Louvain-la-Neuve avec cinq lots différents de milieu conditionné (360 expériences) indique que, sur 18 356 ovocytes récoltés, 13 967 (76 %) se sont clivés dont 3 593 (26 %) ont atteint le stade de blastocyste. Le test d'un même lot de milieu conditionné, au cours de la même période, dans quatre laboratoires différents a donné des résultats divergents : 27 et 37 % de blastocystes/clivés dans deux laboratoires, 7 et 0 % dans les deux autres alors que, dans chaque cas, 30 % de blastocystes avaient été obtenus avec le système de co-colture de référence du laboratoire. Les faibles pourcentages, obtenus dans l'un des laboratoires, peuvent être partiellement expliqués par la toxicité de l'huile utilisée pour recouvrir les gouttes de milieu conditionné ; ce même lot d'huile n'influençant pas le développement en co-culture.

bovin / développement embryonnaire / culture in vitro / milieu conditionné

INTRODUCTION

Oviduct epithelial cells are currently used as culture support for bovine embryos either in co-culture or for conditioning of media (for a review see Gordon, 1994). The drawback of these culture systems is that they contain serum which masks the effect of proteins of cellular origin and which might be embryotoxic (Ménézo, 1983). We therefore conditioned tissue culture medium 199 (TCM 199) with a bovine oviduct epithelial cell (BOEC) monolayer in the absence of serum (Mermillod et al, 1992). This conditioned medium with minimal exogenous protein content (Van Langendonckt et al, 1995) was designed to analyse oviduct secretions and to test their potential effect on embryo development. A similar approach was used by Kobayashi et al (1992) with a serum-free granulosa cell conditioned medium for the culture of bovine embryos and by Liu et al (1995) with human oviduct cell conditioned medium for culturing mouse embryos.

We have shown (Mermillod et al, 1992) that serum-poor BOEC conditioned medium supports early bovine embryo development as well as conditioned medium containing serum, as currently used in other laboratories (Gordon, 1994). Culture conditions were optimized by testing various ratios of embryo number to culture volume (Ferry et al, 1994). The kinetics of embryo development in this medium were characterized by time lapse microcinematography (Grisart et al, 1994). The quality of blastocysts produced in BOEC conditioned medium was assessed by counting their cell number, testing their survival rate after freezing-thawing, scoring hatching rate and finally by investigating their developmental capacity when they are transferred immediately or after cryopreservation (Massip et al, 1995). Metabolic activity, blastocyst cell number and rates of development of embryos produced by co-culture or oviductal conditioned medium were compared (Rieger et al, 1995). Blastocysts produced in conditioned medium had lower cell numbers, developed more slowly and metabolized glucose more precociously than those obtained in co-culture. This indicates that serum-poor BOEC conditioned medium is not the best choice for the production of IVF embryos but should be considered as an experimental tool for the analysis of oviduct secreted factors. A first physicochemical analysis of this medium was performed (Mermillod et al, 1993) and fractionation of putative embryotrophic factors is underway.

In our laboratory, we have routinely used this BOEC conditioned medium for 3 years.

Different batches of medium have been analysed within this period in order to evaluate the reproducibility of the culture system. Medium stability has been tested after long-term freezing and after lyophilization. The same batch of conditioned medium has been tested in four different laboratories. In one laboratory, two types of oil were compared as overlay for the culture drops. A summary of these data is presented in this paper.

MATERIALS AND METHODS

In vitro maturation and fertilization

Oocytes derived from slaughtered cow ovaries were matured and fertilized in vitro according to the method currently applied in the different laboratories (references cited later). All incubations were performed at 39 °C in 5% CO_2 in humidified air.

In Louvain-la-Neuve, Belgium, the method described by Mermillod et al (1993) was used. Briefly, intact cumulus-oocyte complexes (COCs) were aspirated from small follicles, washed in modified Tyrode's medium (containing 6 mg/mL albumin, 4 mg/mL lactate and 0.11 mg/mL pyruvate [Tyrode albumine lactate pyruvate]) and matured for 24 h in TCM 199 (supplemented with 10% FCS, 1 μg/mL β-estradiol, 5 μg/mL pLH and 0.5 μg/mL pFSH). Matured oocytes were washed in TALP and co-incubated for 18 h with 2 x 10^6 spermatozoa mL^{-1} selected after thawing on a Percoll discontinuous density gradient. Fertilization medium was bicarbonate TALP containing 10 μg/mL heparin sodium salt (167 U/mg, Calbiochem, San Diego, CA, USA).

The method applied in the German laboratory is detailed by Berg and Brem (1989). COCs were recovered by puncture, washed in TCM 199 and matured for 24 h in TCM 199 supplemented with 20% estrus cow serum (ECS), 2 mM sodium pyruvate, 2.9 mM calcium lactate, 10 μg/mL FSH, 60 μg/mL gentamycin, 33.9 mM sodium bicarbonate and 4.43 mM Hepes. Fertilization was performed for 20 h using 10^6 frozen-thawed spermatozoa mL^{-1} selected by the swim-up procedure. The fertilization medium was bicarbonate TALP supplemented with 6 mg/mL BSA, 1 μM adrenaline, 10 μM hypotaurine and 10 μg/mL heparin.

In Gent, Belgium, the oocytes were matured and fertilized according to the method of Van Soom et al (1992). Intact COCs were recovered by aspiration of small follicles, washed in modified TCM 199 HEPES containing 20% FCS, 0.2 mM sodium pyruvate, 0.4 mM glutamine and 50 μg/mL gentamycin and matured for 24 h in the same medium, bicarbonate buffered and containing 20% ECS instead of FCS. Matured oocytes were washed in HEPES TALP and fertilized for 20–26 h in bicarbonate TALP (containing 20 μM penicillamin, 10 μM hypotaurine, 1 μM epinephrine, 25 μM heparin) with frozen-thawed spermatozoa selected on a Percoll discontinuous gradient.

In Guelph, Canada, maturation and fertilization were performed as described in Xu et al (1992). COCs were recovered after slicing of the ovary, were washed in TCM 199 supplemented with 10% steer serum, 25 mM HEPES, 2.5 mM sodium pyruvate, 1 mM 1-glutamine and matured for 24 h in the same medium. After maturation, oocytes were washed in HEPES TALP and fertilized with glass-wool filtered frozen-thawed sperm in modified TALP containing 20 μg/mL heparin.

In vitro culture

All the cultures were performed at 39 °C in 5% CO_2 in humidified air except in Munich where a 5% CO_2, 5% O_2 and 90% N_2 gas atmosphere was used.

Culture in BOEC conditioned medium

Cumulus cells of about 100 fertilized oocytes were removed by vortexing for 2 min in 2 mL PBS. Denuded presumptive zygotes were washed in medium 199 and cultured in droplets of conditioned medium (one embryo per μL) under paraffin oil (Merck # 7160, Overijse, Belgium) or silicone oil according to the laboratory.

Control culture systems

In Louvain-la-Neuve, the culture in TCM 199 conditioned with bovine oviduct cells in the presence

of 10% FCS (Eyestone et al, 1991) was used as control.

In Munich, zygotes surrounded by their cumulus were cultured for 3 days in TCM 199 containing 20% ECS; 90 h after fertilization, the embryos were stripped of cumulus cells and further cultured in the same drop (Berg and Brem, 1989). The microdrops were overlaid with paraffin oil and the culture was performed under 5% CO_2, 5% O_2 and 90% N_2 with maximal humidity.

In Gent, the reference culture system was a co-culture with BOEC in B2 medium supplemented with 10% ECS in microdops under silicone oil (Van Soom et al, 1992).

The Canadian reference culture system (Xu et al, 1992) was a co-culture of denuded zygotes with BOEC under filtered silicone oil in modified 199 medium (supplemented with 10% ECS, 6 mg/mL BSA [fraction V], 5 mM sodium pyruvate and 1 mM 1-glutamine).

Evaluation of the culture

The number of cleaved embryos and 5–8 cell stage embryos were counted on day 3 postinsemination (pi) and the number of blastocysts was recorded on days 7, 8 and 9 pi.

Preparation of BOEC conditioned medium without serum supplement

Conditioned medium was prepared according to Mermillod et al (1993). Oviducts were collected at a local slaughterhouse and transported to the laboratory at a temperature of 4 °C. BOEC were isolated by scraping of the oviduct mucosa with a glass slide and were cultured in 25 cm^2 flasks for 4 days in TCM 199 medium supplemented with 10% FCS. The medium was renewed and the cells adhering to the plastic support were cultured for 2 additional days in serum containing TCM 199, until reaching confluence. The cell monolayer was washed three times with serum-free TCM 199 and cultured for 2 days in this medium. After 2 days, the conditioned medium was collected, centrifuged at 500 *g* for 10 min and stored at 4 °C. A second and a third 2 day conditioning were performed on the same monolayer. After three medium collections, the three harvests were pooled.

Medium storage tests

Freezing

The usual storage procedure for conditioned medium was freezing at –80 °C. The medium was stored frozen in 1 mL aliquots in 1.5 mL Ependorf tubes. The aliquots were thawed at room temperature and were centrifuged at 2 000 x *g* for 10 min just prior to use.

Lyophilization

In some experiments, conditioned medium was lyophilized overnight (at –10 °C) in 1 mL aliquots (Leybold-Heraeus GT4 lyophilizator, model TM 220 s2), stored dry at –80 °C and reconstituted with 1 mL of water purified by filtration through a Milli-Q/UF system (Millipore Corporation, New Bedford, MA, USA) and centrifuged just before use. The activity of lyophilized medium was compared with that of untreated medium.

Effect of oil quality on embryonic development in conditioned media or in co-culture

In this experiment, the effect of paraffin oil (used in Louvain-la-Neuve) and silicone oil (used in Gent) on blastocyst yield was compared under different culture systems. After fertilization, denuded zygotes were assigned at random to three culture conditions: culture in serum-poor BOEC conditioned medium, culture in BOEC conditioned medium containing 10% FCS and co-culture with oviduct cells under either silicone oil (Aldrich, #14,615-3, Steinheim, Germany) or paraffin oil (Merck #7160, Overijse, Belgium). The culture was performed in Gent according to the protocol described earlier.

Statistical analysis

Differences in the cleavage rate and blastocyst production rate were analysed by the chi-square test ($P < 0.05$), except in the last experiment, which compared the effect of two types of oil, in which data were analysed with logistic regression.

RESULTS

Stability of conditioned medium

Stability to freezing

Results obtained with one single batch of conditioned medium during the first, second and third year of storage at –80 °C are reported in table I. The mean proportion of cleaved and 5–8 cell stage embryos at 3 days pi and the proportion of blastocysts on day 8 pi did not differ significantly according to the year of medium utilization.

Stability to lyophilization

Results presented in table II indicate that the cleavage rate (on day 3 pi) and the development to the blastocyst stage (on day 9 pi) were not significantly affected by lyophilization of the conditioned medium.

In vitro development in BOEC conditioned medium: test of different medium batches

Five batches of BOEC conditioned medium were extensively tested in the laboratory of Louvain-la-Neuve. A summary of the results obtained in 360 distinct experiments is presented in table III. Only the experiments where an obvious technical problem such as bacterial contamination (phase contrast microscope detection), low fertilization rate (percentage of oocytes with two pronuclei lower than 50%), inappropriate incubator temperature (lower than 38.5 °C or higher than 39.5 °C) occurred were not taken into account (about 5%). Similar developmental rates were obtained with the different batches. On average, 76% of the oocytes cleaved, 51% reached the 5–8 cell stage on day 3 pi and 26% of the cleaved embryos developed into blastocysts.

In vitro development in BOEC conditioned medium: results obtained in four different laboratories with the same batch of medium

One single batch of conditioned medium (9309 BOss) was tested within a 3 month period in four laboratories: Louvain-la-Neuve, Guelph, Gent and Munich. The oocytes were matured and fertilized with the local protocol, and culture in BOEC conditioned medium was performed according to the protocol of Louvain-la-Neuve. In each case, the culture in the conditioned medium

Table I. Mean developmental rates of bovine embryos in a single batch (9309 BOss) of conditioned medium during the first, second and third year of storage (frozen at –80 °C) in experiments carried out at Louvain-la-Neuve, Belgium.

Period	*Oocytes collected* n	*Cleaved/oocytes on day 3* n *(%)*	*5–8 cells/oocytes on day 3* n *(%)*	*Blastocysts/cleaved on day 8* n *(%)*
First year	1 824	1 443 (79)	1 037 (57)	347 (24)
Second year	1 434	1 090 (76)	757 (53)	259 (24)
Third year	1 625	1 251 (77)	790 (49)	327 (26)

Cleavage, 5–8 cell and blastocyst rates do not differ significantly from year to year ($P > 0.05$).

Table II. Number and percentage of cleaved embryos on day 3 pi and the expanded blastocyst stage on day 9 pi in untreated and lyophilized BOEC conditioned medium.

Culture medium	*Oocytes* n	*Cleaved/oocytes on day 3* n *(%)*	*Blastocysts/cleaved on day 9* n *(%)*
Untreated BOEC conditioned medium	191	160 (84)	52 (33)
Lyophilized BOEC conditioned medium	200	153 (77)	55 (36)

Cleaved embryos and blastocyst rates do not differ significantly between untreated and lyophilized conditioned medium ($P > 0.05$).

Table III. Overview of results obtained in Louvain-la-Neuve, Belgium with five different batches of BOEC conditioned medium during a 3 year period.

Conditioned medium batch no	*Oocytes collected* n	*Cleaved/ oocytes on day 3* n *(%)*	*5–8 cells/ oocytes on day 3* n *(%)*	*Blastocysts/ cleaved on day 8* n *(%)*
ov 041 ss	2 420	1 960 (81)	1 379 (57)	459 (23)
ov 174 ss	2 975	2 142 (72)	1 368 (46)	476 (22)
ov 132 ss	6 930	5 198 (75)	3 465 (50)	1 455 (28)
ov 182 ss	4 883	3 760 (77)	2 588 (53)	928 (25)
9309 BOss	1 148	907 (79)	585 (51)	252 (28)
Mean	18 356	13 967 (76)	9 385 (51)	3 593 (26)

No significant difference in cleavage, 5–8 cell stage embryos and blastocyst rates was observed between the different batches ($P > 0.05$).

was compared with the reference culture system of the testing laboratory. Data are shown in table IV. There was no significant difference between the three replicates performed in a given laboratory.

In Guelph, Gent and Munich, the cleavage rate on day 3 pi was not significantly different ($P > 0.05$) between the co-culture and the culture in BOEC conditioned medium.

In these three laboratories, the reference co-culture system yielded more than 30% blastocyst/cleaved embryos. The culture in the conditioned medium yielded similar blastocyst percentage as for the co-culture in Munich but a very poor blastocyst rate as

Table IV. Yields obtained in four different laboratories using oviduct conditioned medium without serum supplement compared to an internal co-culture control. Results are expressed as percentage of cleavage at 3 days pi and % blastocysts/cleaved embryos at 8 days pi.

Culture system *	*Oocytes* n	*Cleaved on day 3* n *(%)*	*Blastocysts/cleaved on day 8* n *(%)*
Louvain-la-Neuve			
BOEC conditioned medium	110	84 (76)	23 (27)
BOEC conditioned medium + serum	117	88 (75)	26 (30)
Guelph			
BOEC conditioned medium	691	391 (57)	28 (7)
Oviduct cell co-culture	750	339 (45)	112 (33)
Gent			
BOEC conditioned medium	96	73 (76)	0 (0)
Oviduct cell co-culture	93	76 (82)	24 (32)
Munich			
BOEC conditioned medium	210	166 (79)	61 (37)
Granulosa cell co-culture	185	139 (75)	66 (47)

* Cultures were performed at 39 °C in 5% CO_2 in humidified air except in Munich where embryos were cultured under 5% CO_2, 5% O_2 and 90% N_2. In each laboratory, three series of experiments were performed. No significant difference was observed between the replicates for each laboratory ($P > 0.05$).

compared to the control co-culture in Gent and Guelph.

Effect of oil quality on embryonic development in conditioned media or in co-culture

The results shown in table V indicate that silicone oil used in combination with both conditioned media had a detrimental effect on bovine blastocyst formation ($P < 0.001$) as compared with paraffin oil but had no significant influence when used in combination with co-culture ($P = 0.95$).

DISCUSSION

There are several reports on in vitro bovine embryo production (for a review see Gordon, 1994), but few data on large-scale embryo production are available except for some reports on commercial bovine embryo yields (Lu and Polge, 1992; Hasler et al, 1995). The present paper gives an overview of the results of culture in serum-poor bovine oviduct conditioned medium, designed for the detection of cell-secreted embryotrophic factors (Mermillod et al, 1993) and examines the applicability of this embryo culture system in four different laboratories.

Our results show that serum-poor BOEC conditioned medium can be stored frozen or lyophilized at –80 °C and may be used for up to 3 years without significant loss of activity. This makes this culture system especially convenient since medium can be prepared in large uniform quantities (up to 200 mL from an oviduct pair, sufficient for 200 embryo cultures) unlike primary co-cul-

Table V. Effect of oil type and culture system on in vitro embryo production.

Culture system	*Oil*	*Oocytes* n	*Cleaved on day 3* n *(%)*	*Blastocysts/cleaved on day 9* n *(%)*
BOEC CM	Silicone	147	118 (80)	1 (1)
BOEC CM	Paraffin	156	114 (73)	9 (8)
BOEC CM + FCS	Silicone	152	116 (76)	3 (3)
BOEC CM + FCS	Paraffin	153	118 (77)	37 (31)
BOEC co-culture	Silicone	253	187 (74)	51 (27)
BOEC co-culture	Paraffin	166	118 (71)	37 (31)

Experiments were carried out at Gent, Belgium. Data were analysed with logistic regression (see Results).

ture where an oviduct cell culture must be initiated for each batch of embryos. Freezing is the most commonly used method of storage for conditioned medium. Eyestone et al (1991) have shown that BOEC conditioned medium containing 10% FCS remains stable after a single cycle of freezing and thawing. The present study demonstrates that serum-poor BOEC conditioned medium is also stable upon freezing without any additives and can be stored for years in this way despite its low protein content (30 µg protein/mL, Van Langendonckt et al, 1995). Lyophilization can also be used for conditioned medium preservation which might be especially convenient for transport. This indicates that factors responsible for the embryotrophic activity of conditioned medium are not destroyed by lyophilization treatment or by freezing in aqueous solution, a property which will facilitate their analysis.

The culture in the serum-poor bovine oviduct conditioned medium yielded an overall blastocyst production rate of 26% blastocysts/cleaved embryos in 360 experiments performed in the laboratory where the culture system was established. Comparison of developmental rates obtained with the same batch of conditioned medium in three external laboratories yielded divergent results: in two laboratories where co-culture yielded blastocyst rates of more than 30%, very low rates were obtained with serum-poor BOEC conditioned medium whereas in another laboratory, results were similar between the co-culture and the conditioned medium. One explanation for this discrepancy could be that embryos cultured in conditioned medium were not protected by the presence of somatic cells and/or serum and therefore could be more sensitive to toxic factors or adverse physicochemical conditions than those co-cultured with oviduct or granulosa cells. Several lines of evidence, as reviewed by Bavister (1995), support the idea that cells and serum may favour embryo development by protecting the embryo against the deleterious effect of oxygen and heavy metals. This hypothesis may hold true under our culture conditions since an atmospheric oxygen concentration was used in three laboratories. This concentration seems to be unfavourable for bovine embryos when cultured without cells (Voelkel and Hu, 1992). This may explain why the best results were obtained in Munich where serum-poor conditioned medium was used in combination with 5% O_2.

Another potentially detrimental component for the embryos cultured in conditioned medium but not for those co-cultured with oviduct cells is the oil overlaying the culture drops. The lower results obtained in both the Gent and Guelph laboratories may be partially explained by the use of silicone oil which was shown in a separate experiment to have negative effects when used in cultures with conditioned medium. Oil can probably release toxic factors into the culture medium and can also alter the composition of culture media by absorbing lipids (Miller and Pursel, 1987). Since oviduct cells continue to produce lipids in culture (Henault and Killian, 1993), this may explain the lack of blastocyst inhibition observed when silicone oil was used in combination with oviduct co-culture. Divergent culture results from different laboratories might also reflect differences in zygote quality (due to the oocyte quality and to the maturation and fertilization procedures) or may be due to the source of chemicals, water or culture dishes which may also be toxic (Bavister, 1995). Unfavourable ambient conditions may have been a factor in the relatively low results obtained in Guelph, where shortly after these experiments were completed, the control co-culture system also collapsed during a particularly cold winter season. Quality controls (eg, media, serum, hormone testing) are critical for accurate and reproducible data in IVF programs (Schiewe et al, 1990) and are applied in the laboratories involved in the present study. However, despite these precautions, our data seem to indicate that conditions optimized for one culture system can be inadequate for another.

The main conclusion that can be drawn from this report is that a culture system (using serum-poor BOEC conditioned medium) which was shown to yield reproducible development rate over a long period in a given laboratory can yield divergent results in other laboratories.

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