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In vitro development of bovine embryos cultured under different fetal calf serum concentrations and cell types

Desenvolvimento in vitro de embriões bovinos cultivados sob diferentes concentrações de soro fetal bovino e tipos celulares

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Abstract

The present work was carried out to evaluate the *in vitro* development of bovine embryo co-cultured in granulosa, oviduct, BRL or VERO cells co-cultures, supplemented with 5% or 10% of Fetal Calf Serum (FCS). Cummulus oocyte complexes were aspirated, matured and fertilized *in vitro*. Embryonic structures were divided into eight treatments. They were placed in culture media TCM 199 containing granulosa, oviduct, BRL or VERO cells, each of them added with 5% or 10% FCS. The conditions for the co-culture were 38.5 ºC, 5% CO_2 in air and high humidity for ten consecutive days. Cleavage, blastocyst and hatching rates did not differ ($p > 0.05$) in co-culture with primary cells (granulosa and oviduct) when FCS concentration increased from 5 to 10%. However, in continuous cells co-culture (BRL and VERO), when FCS concentration increased from 5% to 10%, the blastocyst development rate decreased significantly ($p < 0.05$) from 33.6 to 16.3% and from 40 to 16.5% in embryo co-culture with BRL and VERO cells, respectively.

Keywords: Co-culture. Blastocyst. Granulosa. Oviduct. Vero. BRL.

Resumo

O presente trabalho foi realizado com o objetivo de avaliar o desenvolvimento *in vitro* de embriões bovinos co-cultivados em células da granulosa, do oviduto, BRL e VERO, suplementados com 5% ou 10% de Soro Fetal Bovino (SFB). Os complexos cummulus oócitos foram aspirados, maturados e fecundados *in vitro*. As estruturas embrionárias foram divididas em oito tratamentos: co-cultivo em TCM 199 contendo células da granulosa, do oviduto, BRL ou VERO adicionadas com 5% ou 10% de SFB. As condições de cultivo foram 38.5 ºC, 5% CO_2 em ar e alta humidade por dez dias consecutivos. Os índices de clivagem, blastocisto e eclosão não diferiram ($p > 0,05$) no co-cultivo com células primárias (granulosa e oviduto) quando a concentração de SFB aumentou de 5 para 10%. Entretanto, no co-cultivo com células de linhagens contínuas (BRL e VERO), quando a concentração de SFB aumentou de 5% para 10%, os índices de blastocistos diminuíram significativamente ($p < 0,05$) de 33,6 para 16,3 % e de 40 para 16,5% nos embriões bovinos co-cultivados com células VERO e BRL, respectivamente.

Palavras-chave: Co-cultivo. Blastocisto. Granulosa. Oviduto. Vero. BRL.

Introduction

Somatic cell co-culture plays a major role on development of preimplantation embryos cultured in vitro[1]. The embryotrophic effects of co-culture are mediated by somatic cell production of growth and survival factors as well as metabolism of inhibitory substances such as reactive oxygen species[1,2,3,4,5]. Nonetheless, inadequate culture system will favor both the embryonic blockage and loss[6].

It has been demonstrated that primary oviductal (OC)[7,8,9] and granulosa cell (GC)[10,11] culture secretes a series of factors that act in a paracrine manner stimulating development to the blastocyst stage. Moreover, it was shown that co-culture of embryos with these cells types was beneficial due to embryotrophic effects of secretions of cultured cells as well as due to the removal of embryotoxic substances from the cul-

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ture medium[12]. Similarly, permanent cell lines, such as Buffalo rat liver cells (BRL), produce considerable levels of insulin-like growth factor-II (IGF-II)[13], transforming growth factor beta (TGF-β)[14] and Leukemia inhibitory factor (LIF)[15], while african green monkey kidney (VERO) cell, a safe pathogen-free epithelial cell (they are highly controlled for viruses and other contaminants because they are used for vaccine production) produce interleukin, platelet-derived growth factor (PDGF), LIF and IGF[16] promoting an stimulatory effect during embryonic development[17,18,19,20,21].

Even though the beneficial effect of co-culture on development of *in vitro* produced embryos has been widely demonstrated there is controversial evidence regarding domestic animal species cell specificity and embryo co-culture. Therefore, this study was carried out to evaluate the effect of GC, OC, BRL or VERO cell co-culture systems supplemented with 5 or 10% fetal calf serum (FCS).

Material and Method

Chemicals and Reagents. Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Tissue culture medium (TCM)-199 and fetal calf serum (FCS) were obtained from Gibco BRL (Grand Island, NY, USA).

Collection of Oocytes. Slaughterhouse bovine ovaries were transported to the laboratory in thermal recipients containing 0.9% NaCl saline solution at 30 ºC, washed twice in phosphate buffered saline (PBS, Nutricell – Brazil) and maintained at 30 ºC water bath. Cumulus-oocyte complexes (COCs) were obtained by aspirating 2-8 mm follicles with a 20-gauge needle coupled to a 10 mL syringe. The follicular fluid was pooled in 15 mL conical tubes and allowed to sediment for 10–15 min.

In vitro Maturation. Cumulus-oocyte complexes (COCs) containing compact cumulus cells and homogeneous/brown cytoplasm were selected for *in vitro* maturation. Selected COCs were washed three times in washing medium (TCM 199-Hepes + 10% FCS + 22 µg/mL pyruvate + 50 µg/mL gentamicin) and twice in maturation medium (TCM 199 + 10% FCS + 0.5 µg/mL FSH + 5 µg/mL LH + 1 µg/mL E_2 + 22 µg/mL pyruvate + 50 µg/mL gentamycin). Groups of 30 COCs were matured in 90 µL drop maturation medium under mineral oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air for 22-24 hours.

Sperm preparation and In vitro fertilization. COCs were removed from maturation drops and washed once in TCM-HEPES supplemented with 3 mg/ml BSA + 22 µg/mL pyruvate + 50 µg/mL gentamycin. Frozen-thawed semen was purified by centrifugation in Percoll gradient (45% and 90%) to separate viable spermatozoa. Groups of 20-30 COCs were placed in 90 µL drop fertilization medium (FERT-TALP + 3 mg/ml BSA + 22 µg/mL pyruvate + 50 µg/mL gentamycin) supplemented with PHE [0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl] and heparin (100 UI/mL).

Oocytes were inseminated with 1 x 10^6 spermatozoa/mL at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. Approximately 18 h post-insemination presumptive zygotes were removed from fertilization drops and denuded of cumulus cells by vortexing in TALP medium. Putative zygotes were washed twice in fertilization medium and three times in culture medium.

Embryo Culture. Groups of 30 presumptive zygotes were randomly allocated in 250 µL TCM-199 containing one of the following co-culture systems: granulosa cells + 5% FCS, granulosa cells + 10% FCS, oviductal cells + 5% FCS, oviductal cells + 10% FCS, BRL cells + 5% FCS, BRL cells + 10% FCS, VERO + 5% FCS or VERO + 10% FCS underlaid in mineral oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. At day 2 postinsemination embryos were supplemented with 250 µl culture medium and cleavage rate was evaluated. Blastocyst and hatched blastocyst rates were determined at day 10 postinsemination.

Somatic Cell Co-Culture.

Granulosa cells (GC)

Slaughterhouse ovaries were washed in PBS containing antibiotic solution. Follicular fluid was aspirated from 2-8 mm follicles using a 5-mL syringe coupled with a 21-gauge needle. The follicular fluid was pooled in 15 ml conical tubes and allowed to sediment for 10–15 min. The precipitate was collected and deposited in a sterile Petri dish in order to remove the oocytes under a stereomicroscope. The cell pellet remaining was washed once in PBS, and three times in TCM-199 by sedimentation. The pellet was resuspended in TCM 199 containing 10% FCS and placed in NUNC 4 well plates (500 µL/well) under silicone oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air for two days until confluence was achieved. Approximately 1 x 10^3 cells were co-cultured in NUNC 4-well plates (500 µL/well) under mineral oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Oviductal Cell (OC)

Slaughterhouse oviducts were obtained from cows with presence of a corpus luteum on the ovary. Oviducts were rinsed twice [70% ethanol and PBS supplemented with 10% FCS (PBS-FCS)], dissected of connective tissue and rinsed in PBS-FCS. Epithelial cells were collected by stripping the oviduct from isthmus to infundibulum. Cells were placed into a 15 ml conical tube and the pellet was washed once in PBS and three times in TCM-199 by sedimentation. The pellet was resuspended in TCM-199 supplemented with 10% FCS + 22 µg/ml pyruvate + 22 µg/ml gentamycin. Cells were cultured in NUNC 4-well plates (500 µl/well) under silicone oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air for two days until confluence was achieved. Approximately 1 x 10^3 cells were co-cultured in NUNC 4-well plates (500 µl/well) under mineral oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Buffalo rat liver (BRL) and African green monkey kidney (VERO) cells

Frozen Buffalo rat liver (obtained from Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Faculdade de Ciências Agrárias e Veterinárias, UNESP-Jaboticabal) or Vero cells (obtained from Department of Animal Science, College of Agriculture and Natural Resources, University of Connecticut-Storrs) were thawed and cultured in modified Dubecco supplemented with 10% FCS + 22 µg/mL pyruvate + 22 µg/mL gentamycin at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air for 24 until confluence. Confluent cultures were dispersed in 0.1% trypsin in calcium and magnesium-free PBS, placed into a 15 mL conical tube and washed once in PBS by centrifugation (400 g for 5 minutes). The cell pellet was resuspended in TCM-199 supplemented with 10% FCS + 22 µg/mL pyruvate + 22 µg/mL gentamycin. Approximately 1 x 10^3 cells were co-cultured in NUNC 4-well plates (500 µL/well) under mineral oil at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Statistical Analysis. Statistical data analysis was performed by using the application Guided Data Analysis and Analyst of the SAS System for Windows V8 (SAS Institute Inc., Cary, NC, USA, 2000)[22]. Data were tested for normality of residues and homogeneity of variances and when normality could not be achieved, the NPAR1WAY procedure for non-parametric variance analysis was employed. Treatment differences $P < 0.05$ were considered significant.

Cleavage rate was calculated on total of oocytes that undergo *in vitro* fertilization. Blastocyst rate was calculated on cleavage, and blastocyst hatching on blastocyst number.

Results

A total of 4266 oocytes were subjected to *in vitro* fertilization, which were further cultured up to ten

days in eight different treatments. The overall results of *in vitro* embryo development of the presumptive zygotes cultured upon the monolayer of garanulosa, oviduct, BRL or VERO cells supplemented with 5 or 10% of FCS are shown in the table 1. There was no difference in cleavage rate among the eight groups. Similarly, there was no difference in the overall hatched blastocyst yield at Day 10. However, the blastocyst rates were significantly affected by culture system (P < 0.05).

Regards FCS effects on embryo development in primary cell line (OC and GC), no significant difference was found in blastocyst rates cultured in granulosa or oviduct monolayer when FCS concentration increased to 10 %. In the other hand, blastocyst rates were significantly affected by FCS concentration in co-culture with continuous cell line. Embryos cultured in BRL monolayer supplemented with 10% FCS were less likely to develop to the blastocyst stage (16.3%) than embryo cultured in BRL monolayer supplemented with 5% FCS (33.6%; P < 0.05). Similar result was found in embryos cultured with VERO monolayer, which blastocyst rate was significantly reduced by increase of FCS concentration to 10% (P < 0.05).

Comparing the embryo development among the different cell type co-culture (garanulosa, oviduct, BRL or VERO) supplemented with 5% of FCS, no significant difference was observed in the blastocyst rate (P > 0.05). However, when different cell monolayer were supplemented with 10% of FCS, The blastocyst rate decrease from 26.1 % in granulosa cell co-culture to 16.3 % and 16.5 % in BRL and VERO cell co-culture respectively.

Table 1 - Effect of supplementation with 5 or 10% fetal calf serum (FCS) in different cell monolayer co-culture on *in vitro* bovine embryo development

Cell	FCS (%)	Oocytes N	Cleaved N (%)	Blastocysts N (%)	Hatched Bl. N (%)
Granulosa	5	371	303 (81.7)	125 (41.3)[a]	79 (63.2)
	10	901	582 (64.6)	152 (26.1)[a]	117 (76.9)
Oviduct	5	373	281 (75.3)	91 (32.4)[a]	46 (50.5)
	10	653	442 (67.7)	109 (24.7)[ab]	88 (80.7)
BRL	5	360	274 (76.1)	92 (33.6)[a]	64 (69.6)
	10	626	412 (65.8)	67 (16.3)[c]	42 (62.7)
VERO	5	364	260 (71.4)	104 (40.0)[a]	70 (67.3)
	10	618	443 (71.7)	73 (16.5)[bc]	33 (45.2)

Hatched Bl. = hatched blastocyst; N = Number; BRL = buffalo rat cell; VERO = African green monkey kidney cell. [abc]Values followed by different superscript letters are significantly different (p < 0.05)

Discussion

The higher FCS concentration compromised BRL and VERO cell co-culture efficiency reducing blastocyst rate. This decrease in blastocyst rate may be due to FCS-induced embryonic morphological alterations. Several studies have demonstrated that lipid accumulation often occurs in ruminant embryos cultured in the presence of serum[23,24,25]. For example, morulae and blastocysts cultured in TCM199 supplemented with serum carry a large number of high density lipid droplets suggesting the presence of poliunsaturated fatty acids (PUFA)[26]. This increase in PUFA, which has been described down-regulate mRNA expression of proteins involved in lipid metabolism[27], can predispose the embryo to oxidative injuries triggering lipid

peroxidation chain reaction and consequent membrane damage[28,29]. Alterations in membrane function and permeability, especially in mitochondrial membranes, may cause irreversible loss of cellular respiration, oxidative phosphorylation and ion transport[30] compromising embryonic metabolism[29,31] and messenger RNA expression[32]. Moreover, serum supplementation has been shown to increase the number of immature mitochondria[16], inhibit cell division[33,34] and induce mitochondria-mediated apoptosis[35]. FCS also increases the percentage of apoptosis reducing the number of cells per blastocyst[36] and decreases the blastocyst rate[37].

The negative effect caused by higher FCS concentration was not observed in GC or OC co-culture. It is possible that these cells metabolized additional FCS components reducing embryonic oxidative stress. Somatic cell co-culture may reduce glucose concentrations in the culture medium reducing glucose toxicity in early embryos[38]. For instance, OC co-culture has been shown to produce embryotrophic factors[7,39], reduce O_2 tension[40,41], glucose concentration[41,42] and suppressed apoptosis in addition to stimulating proliferation of blastomere[43]. Moreover, GC and OC behaved similarly for cleavage rate and embryo development[44].

In the current study the co-culture efficiency variation may be due to the inherent characteristics of primary (GC and OC) versus cell line cultures (BRL and VERO). Cellular growth requirements are more complex in primary culture as compared to continuous cell lines. This is likely because many cell lines are derived from tumor tissues and have reduced growth factor need. In contrast, primary cells require a wide range of supplements such as adherence factors, collagen (types I, II and IV), fibronectin, insulin, IGF-II, IL-2, IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), endothelial cell growth supplement (ECGS), parathyroid hormone, fibroblast growth factor (FGF), epidermal growth factor (EGF), hydrocortisone, FSH, nerve growth factor (NGF), estrogens and testosterone[45]. In addition, the differences between primary and continuous cell monolayer behavior in co-culture may also explain the reduction in blastocyst rate in VERO and BRL cells co-cultures supplemented with 10% FCS, which primary culture have contact-inhibition and topoinhibition (density growth inhibition), whereas continuous cell lines do not[46].

Moreover, Maeda et al.[47] demonstrated that blastocyst development was greater for embryos cultured in GC conditioned medium or co-culture as compared to VERO conditioned medium or co-culture. While GC conditioned cell secreted 32-37 μg/mL total protein, VERO cells secreted 2-4 μg/mL total protein. Such secretory activity may explain stimulatory effect of GC co-culture on blastocyst rate. Similar protein concentration (35.6 μg/mL) was also observed by Kobayashi et al.[48] in TCM199 medium conditioned by GC, while Mermillod et al.[49] obtained a final protein concentration of 40 μg/mL in the medium conditioned with OC. This increased protein production by OC and GC cells may have reduced serum requirement during co-culture and/or inhibit the detrimental effect of high FCS concentration.

Other factors to be considered are the culture medium energy sources. For instance, BRL cell line require relatively high glucose concentrations in order to present optimum growth conditions, whereas embryos need low concentrations at the beginning of embryonic development and higher concentrations after compaction stages[50,51]. Thus, the competition for the medium glucose source between embryo and BRL monolayer, might explain why there was no difference in the cleavage rate, when the embryo preferably consumes pyruvate, but there was difference in the blastocyst rate, when the embryo consumes glucose. The mechanism by which primary cell lines, or more specifically granulosa and oviduct cells, are capable of reducing the deleterious effects of the FCS high con-

centrations on embryos is still unclear, deserving further attention.

Conclusion

Supplementation with 5 or 10% of FCS did not influence the embryonic development in granulosa or oviduct cells co-culture. The increase of FCS concentration to 10% in BRL or VERO cell co-culture, however, decreased the blastocyst rate. These results show that continuous cell lines under high FCS concentration has different behavior from primary cell in bovine embryo co-culture. In this way, bovine embryo co-cultured with primary cells (granulose or oviduct) supplemented with 5% or 10% FCS or co-cultured with continuous cells line (BRL or VERO) supplemented with 5% FCS concentration are the system that better support the embryo development to the blastocyst stage.

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