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Is the early reduction of fetal calf serum concentration in bovine *in vitro* embryo culture beneficial?

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Abstract

This study was designed to investigate the effect of supplementation with 5 or 10% fetal calf serum (FCS) on bovine embryo development in three types of culture media added with amino acids (aa): Charles Rosenkrans 2 medium (CR2aa), potassium simplex optimized medium (KSOMaa) and synthetic oviduct fluid (SOFaa). *In vitro* matured (IVM) and fertilized (IVF) oocytes were *in vitro* cultured (IVC) from Day 1 to 10 of development. The increase in FCS in CR2aa media did not affect embryo development and hatching. However, the increase from 5 to 10% FCS in KSOMaa and SOFaa induced a decrease in the number of blastocysts. Embryo hatching was similar for embryos cultured with 5 or 10% FCS in both media. We concluded that a lower concentration of FCS increased embryo development rate in SOFaa and KSOMaa media and that there was no detrimental effect of high FCS concentration in CR2aa.

Keywords: embryo development, bovine embryos, fetal calf serum, in vitro culture.

Introduction

The successful development of *in vitro* produced (IVP) bovine embryos depends on environmental factors during the stages of *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC; Bracket and Zuelke, 1993). In *in vitro* embryo production, the culture system may deeply influence embryonic development (Lonergan *et al*., 1999). It is well established that embryo metabolism, cleavage, and pregnancy rates are affected by the media composition, which may lead to a diminished embryonic and fetal developmental capacity (Eckert *et al*., 1998).

Early works on *in vitro* embryo development contributed to the improvement of IVC protocols, resulting in the development of defined media (Tervit *et al*., 1972; Lawitts and Biggers, 1991; Rosenkrans *et al*., 1993). Despite the fact that bovine embryos can be cultured *in vitro* in simple media under defined conditions (Holm *et al*., 1999), the beneficial effects of fetal calf serum (FCS) on the development of preimplantation bovine embryos have been demonstrated (Gómez and Diez, 2000). Embryos cultured in the absence of proteins displayed changes in metabolic activity (Eckert et al., 1998), reduced development (Thompson et al., 1998), and in cell number (Lim et al., 1996) when compared to embryos cultured in the presence of proteins.

The requirement for biological components such as FCS or bovine serum albumin (BSA) in IVP is a matter of discussion due to their complex and undefined compositions (Gardner and Lane, 1993). Fetal calf serum has been shown to increase membrane permeability, decrease transepithelial electrical resistance, and increase gap junction permeability in cultured cells (Mortell *et al*., 1993). Also, FCS has been shown to increase the number of embryos that reach the blastocyst stage as well as the proportion of hatched blastocysts.

Despite the common use of several culture media for IVP embryos, the comparisons among supplementation with different FCS concentrations in different media with *in vitro* culture have not been systematically tested. This study evaluated the effect of different FCS concentrations in three (CR2, KSOM, and SOF) embryo culture media on bovine embryo development.

Materials and Methods

Oocyte collection

Ovaries were obtained from slaughtered cows and washed twice in PBS at 30°C to remove blood and debris. Cumulus-oocyte complexes (COCs) were aspired from 2- to 8-mm follicles using a 25-G needle coupled to a 10-ml syringe. The follicular fluid was placed in a 15-ml conical tube for 10 to 15 minutes. The precipitate was collected and deposited in a sterile Petri dish and oocytes were localized under a stereomicroscope.

Oocyte maturation

Cumulus-oocyte complexes with compact cumulus cells and homogeneous cytoplasm were washed three times in holding medium (TCM 199-Hepes + 10% FCS + 0.2 mM pyruvate + 50 μg/ml.

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gentamycin) and twice in maturation medium (TCM 199 + 10% FCS + 0.5 µg/ml FSH + 5 µg/ml LH + 1 µg/ml E2 + 0.2 mM pyruvate + 50 µg/ml gentamycin). Groups of 20 to 30 COCs were placed in 90-µl drops of maturation medium overlaid with mineral oil and incubated at 38.5°C in 5% CO_2 and 100% humidity for 22 to 24 hours.

In vitro fertilization

After *in vitro* maturation, COCs were washed three times in holding medium and transferred in groups of 20 to 30 into 90 µl of in vitro fertilization (IVF) medium (Talp + 3 mg/ml BSA + 0.2 mM pyruvate + 50 µl/ml gentamycin added of 0.25 mM epinephrine, 1 mM hypotaurine, penicillamine and 2 mM heparin) overlaid with mineral oil. Cryopreserved semen was thawed in a water bath at 37°C and centrifuged in a Percoll gradient (45% and 90%) at 600 x *g* to separate the viable spermatozoa. Oocytes were fertilized with 1 x 10^6 spermatozoa/ml at 38.5°C in 5% (v/v) CO_2 and 100% humidity for 18 hours.

Embryo culture

After fertilization, cumulus cells were mechanically removed and putative zygotes were randomly distributed in 4-well plates (NUNC®) containing 500 µl of the culture media CR2aa, KSOMaa, or SOFaa, supplemented with 5% or 10% FCS (Day 1) per well. Presumptive zygotes were cultured at 38.5°C in 5% (v/v) CO_2 and 100% humidity for 10 days. On Day 3, culture wells were supplemented with 250 µl of fresh media and the cleavage rates were evaluated. Blastocyst and hatched blastocyst stages were determined at Days 7 and 10 post-fertilization, respectively.

Statistical analysis

For statistical analysis, the Minitab Release 13.0 (Minitab Inc., State College, PA, USA) was used and the data were subjected to non-parametric analysis by using the Mann–Whitney tests. The results for cleavage, blastocyst, and hatching rates obtained for each FCS concentration (5 or 10%) in different culture media (CR2aa, KSOMaa, and SOFaa) were expressed as percentage. The level of significance was $P < 0.5$.

Results

The increase in FCS concentration from 5 to 10% in CR2aa medium reduced the cleavage rate ($P < 0.05$). However, there were no significant differences in blastocyst development ($P > 0.05$) and blastocyst hatching ($P > 0.05$) between embryos cultured with 5 or 10% of FCS (Table 1).

Table 1. Effect of supplementation with 5 or 10% fetal calf serum (FCS) in CR2aa media on *in vitro* development of bovine embryos.

FCS (%)	Oocytes Fertilized (n)	Cleavage (n;%)	Blastocyst Rate (n; %)	Hatched Embryos (n; %)
5	427	356 (83.37) [a]	170 (39.81)	141 (33.02)
10	336	219 (65.17) [b]	115 (34.23)	69 (20.54)
P – value		0.02	0.11	0.81

Blastocyst and hatching rate were calculated in relation to the percentage of oocytes that were fertilized. Within columns, values with different letters are significantly different ($P < 0.05$).

With KSOMaa medium, the cleavage rate was similar for zygotes cultured in either 5 or 10% of FCS ($P > 0.05$). While the proportion of blastocysts was significantly greater ($P < 0.05$) when embryos were cultured in KSOMaa supplemented 5% compared to 10% of FCS, the hatching rate was not significantly different ($P > 0.05$) between the groups as showed in Table 2.

Table 2. Effect of supplementation with 5 or 10% fetal calf serum (FCS) in KSOMaa media on *in vitro* development of bovine embryos.

FCS (%)	Oocytes Fertilized (n)	Cleavage (n; %)	Blastocyst Rate (n; %)	Hatched Embryos (n; %)
5	422	308 (72.98)	172 (40.75)[a]	145 (34.36)
10	278	193 (69.42)	73 (26.26)[b]	64 (23.02)
P - value		0.96	0.03	0.74

Blastocyst and hatching rate were calculated in relation to the percentage of oocytes that were fertilized. Within columns, values with different letters are significantly different ($P < 0.05$).

Similar results were observed in SOFaa medium (Table 3). Different FCS concentrations did not affect cleavage rate ($P > 0.05$). When the FCS concentration increased from 5 to 10%, the blastocyst rate was significantly reduced ($P < 0.05$). However, there were no significant differences ($P > 0.05$) in blastocyst hatching between embryos cultured and supplemented with different FCS concentrations.

Table 3. Effect of supplementation with 5 or 10% fetal calf serum (FCS) in SOFaa media on *in vitro* development of bovine embryos.

FCS (%)	Oocytes Fertilized (n)	Cleavage (n; %)	Blastocyst Rate (n; %)	Hatched Embryos (n; %)
5	421	351 (83.37)	226 (53.68)[a]	130 (30.87)
10	302	243 (80.46)	85 (28.15)[b]	66 (21.85)
P - value		0.49	0.01	0.37

Blastocyst and hatching rate were calculated in relation to the percentage of oocytes that were fertilized. Within columns, values with different letters are significantly different ($P < 0.05$).

Discussion

The reduction to 5% FCS during *in vitro* culture resulted in a higher blastocyst rate in KSOMaa and SOFaa media. This increase in blastocyst rate when FCS concentration was reduced in SOFaa culture media was described earlier (Assumpção *et al*., 2002). The decrease in blastocyst rate in culture medium supplemented with higher FCS concentrations may be due to morphological alterations provoked by the FCS. Several studies have demonstrated lipid accumulation in ruminant embryos cultured in media supplemented with serum (Dorland *et al*., 1994; Thompson *et al*., 1995; Abe *et al*., 1999b; Reis *et al*., 2003). Morulae and blastocysts developed in TCM199 supplemented with bovine serum (BS) had a large number of high density lipid droplets suggesting unsaturated fatty acids, whereas those cultured in BS-free medium had large vesicles, resembling lysosomes but with fewer lipid droplets similar to *in vivo* developed morulae and blastocysts (Abe *et al*. 1999a, b).

Unsaturated fatty acids can induce oxidative injuries in mammalian embryos (Nonagaki *et al*., 1994). Peroxidation of these lipids can cause structural damage, affecting membrane function and permeability especially in mitochondrial membranes and lead to irreversible loss of functions such as respiration, oxidative phosphorylation, ion transport (Kowaltowski and Vercese, 1999), and altered embryo metabolism (Johnson and Nasr-Esfahani, 1994).

The increase in blastocyst rate for culture in KSOMaa and SOFaa supplemented with 5% FCS may be due to a lower lipid droplet accumulation and fewer alterations in mitochondrial patterns. This leads to slight alterations in energy metabolism, providing a better energy utilization to achieve higher blastocyst rates when compared to embryos cultured in medium with 10% FCS.

The medium CR2aa did not yield a significant difference in blastocyst rate when cultured with 5% or 10% FCS. This was probably due to a higher pyruvate concentration in CR2aa medium (2.0 M) in relation to SOFaa (0.30 mM) KSOMaa (0.5 mM), and oviduct physiological concentration (0.5 mM; Leese, 1988). Apparently, the amount of pyruvate added to the medium does not influence embryonic metabolism. However, among the mitochondrial alterations provoked by FCS, there is a significant presence of a large amount of immature mitochondria similar to early embryo stages (Abe *et al*. 1999b). Nevertheless, ATP production and glucose consumption is low in immature mitochondria of early embryos. Pyruvate and lactate, the "fuel" for the citric acid cycle (CAC), are predominant during these stages (Thompson *et al.,* 1992, 1996; Gardner *et al*., 1993).

Thus, the addition of 10% of FCS in culture media may have provoked the increase in immature mitochondria in later stage embryos, consuming more pyruvate than embryos with mature mitochondria. Most likely, the CR2aa medium, which had the greatest pyruvate concentration, might have been used by these immature mitochondria and increased energy production and supported embryonic development.

Another assumption on how the greatest pyruvate concentration might have reduced the damages provoked by the increased serum concentration in CR2aa medium compared to the other media may be the pyruvate's capacity to inhibit apoptosis triggered by oxidative stress. Free radicals produced by serum in embryonic *in vitro* culture reduce the number of cell divisions (Crosier *et al*. 2000, 2001) and regulate mitochondria-mediated apoptosis (Liu *et al*., 2000). Moreover, serum induces an increase in apoptosis incidence and reduction in blastocyst cell number in *in vitro* produced bovine embryos (Byrne *et al*., 1999; Reis *et al*., 2003). Pyruvate acts as an antioxidant in cells, inhibiting reactive oxygen species (ROS) by changes in cellular redox state. It has been proved that pyruvate has the capability of eliminating hydrogen peroxides (H_2O_2) and other ROS (Bassenge *et al*., 2001). In addition,

pyruvate inhibits the activation and translocation of p53, followed by damages caused by H_2O_2, modulating bcl-2 and bax gene protein expression and maybe other components of the apoptotic death cascade. Pyruvate may also help to facilitate cytosolic and mitochondrial tolerance against apoptosis triggers (Lee *et al*., 2003).

In conclusion, the reduction of FCS in KSOMaa and SOFaa media was beneficial for *in vitro* embryo culture, resulting in higher blastocyst rates and that the deleterious effect of high FCS concentrations is dependent of culture media used.

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