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The effects of ovalbumin as a protein source during the *in vitro* production of bovine embryos

Tatiane Almeida Drummond Tetzner[1], Naiara Zoccal Saraiva[1], Felipe Perecin[2], Simone Cristina Méo Niciura[3], Christina Ramires Ferreira[4], Clara Slade Oliveira[1], Joaquim Mansano Garcia[1]

[1] *Departamento de Reprodução Animal, FCAV, UNESP, Jaboticabal, Brasil.*
[2] *Faculdade de Zootecnia e Engenharia de Alimentos, FZEA, USP, Pirassununga, Brasil.*
[3] *Embrapa Pecuária Sudeste, São Carlos, Brasil.*
[4] *Departamento de Química Orgânica, Instituto de Química, UNICAMP, Campinas, Brasil.*

ABSTRACT - Embryo quality is influenced by the culture conditions that affect *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) rates. The present study investigated the feasibility of producing bovine embryos after the replacement of fetal calf serum (FCS) and bovine serum albumin (BSA) by ovalbumin (OVA). The IVM and IVC medium were supplemented with 10% FCS, 4 mg/mL BSA, or 4 mg/mL OVA. The IVF medium was supplemented with 6 mg/mL BSA or OVA. For IVM, supplementation with FCS, BSA, and OVA did not affect nuclear maturation or cortical granule migration. Higher rates of formation of two pronuclei were obtained when FCS was employed for IVM (79.97%), regardless of the supplement used for IVF, and when BSA was used for IVF (59.4%), regardless of the supplement used for IVM. Supplementation with OVA for IVM+IVC (20.40%) and for IVF (22.15%) was inferior to supplementation with FCS for IVM+IVC (30.47%) and with BSA for IVF (28.91%) for blastocyst development. Hatching rates were lower using OVA for IVM+IVC (23.02%) and for IVF (28.93%) compared with FCS and BSA under the same conditions (40.78 and 34.82%, respectively) and BSA for IVF (36.82%). Supplementation with OVA for IVM+IVC and IVF resulted in reduced inner cell mass, trophectoderm cells and total blastocyst cell numbers (17.29, 37.88, and 55.17, respectively). In conclusion, OVA is a protein source for bovine *in vitro* embryo production, although the quantity and quality of bovine blastocysts using only ovalbumin in the entire *in vitro* production process are lower than those obtained in the presence of FCS and BSA, when used as supplements in any step of bovine *in vitro* embryo production.

Key Words: bovine embryo, *in vitro* culture, ovalbumin, protein source

Introduction

In vitro production of bovine embryos enables greater reproductive use of genetically superior animals and reduces the interval generation, improving animal breeding. Thus, studies investigating the three steps of *in vitro* embryo production (IVP) are necessary to try to achieve results comparable to those obtained *in vivo*.

The culture media used for mammalian embryo development are conventionally supplemented with fetal calf serum (FCS) or bovine serum albumin (BSA) (Bavister et al., 1992; Gardner & Lane, 1993). FCS and BSA are complex, undefined mixtures of different proteins, which contain small peptides, energy substrates and growth factors (Chaudhry et al., 2008, Kane & Headon, 1980; Pratten et al., 1988). However, because BSA and FCS are prepared and purified from bovine blood products, they present a high risk of contamination by pathogens, viruses (Guerin et al., 1988), and prions (Batt et al., 1991; Krisher et al., 1999). Thus, alternative supplements have been evaluated to replace BSA and FCS during IVP in order to avoid cross-contamination. The most commonly defined supplements used for IVP are synthetic macromolecules, such as polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) (Takahashi & First, 1992; Wrenzycki et al., 1999). However, the results are controversial, and the quantity and quality of the embryos produced are often unsatisfactory.

Therefore, in the present study, ovalbumin (OVA), a protein source that has not been previously reported for bovine embryo IVP was evaluated. Ovalbumin, the main protein component of albumen (54%), is a phosphoprotein whose synthesis is stimulated by estrogen (Bahr & Johnson, 1991). Ovalbumin contains all essential amino acids (Besler & Mine, 1999). Thus, OVA is a protein supplement that can maintain cell proliferation (Barlian et al., 1993). In addition, the risks of transmission of diseases to cattle are lower because of the heterologous (avian) origin of OVA.

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Corresponding author: tatianevet@hotmail.com

The objective of the present study was to compare the effects of OVA and bovine blood proteins (FCS and BSA) on the IVP. The effects of OVA on nuclear and cytoplasmic maturation, pronuclear formation, embryo cleavage rates, blastocyst development, hatching rates and numbers of cells were carefully investigated.

Material and Methods

Chemicals and media were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Bovine oocytes were collected by follicular aspiration from ovaries obtained at a local slaughterhouse and transported to the laboratory in 0.9% saline at 30-35 ºC. Follicles measuring 3 to 8 mm in diameter were aspirated with an 18-gauge needle connected to a 20-mL syringe. Cumulus-oocyte complexes with at least three cell layers and homogenous cytoplasm were selected and washed in HEPES-buffered tissue culture medium 199 (TCM-199; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FCS (Crypion, Andradina, Brazil), 0.20 mM sodium pyruvate, and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil).

For IVM, groups of 40-50 oocytes were cultured in 400 µL of TCM-199 containing Earle's salts (Gibco-BRL), 1.0 µg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 IU/mL hCG (Profasi™, Serono, São Paulo, Brazil), 1.0 µg/mL estradiol, 0.2 mM sodium pyruvate, and 83.4 µg/mL amikacin under mineral oil (Dow Corning Co., Midland, MI, USA) in 4-well dishes (Nunc, Rochester, NY, USA) for 24 h at 38.5ºC in 5% CO_2 in the air. The IVM medium was supplemented with either 10% FCS, 4 mg/mL BSA, or 4 mg/mL OVA.

After IVM, oocyte maturation was evaluated based on the stage of nuclear progression (MII stage) and cortical granule migration to the periphery of the cytoplasmic membrane. For this purpose, 1,505 oocytes were divided into three experimental groups and analyzed in quadruplicate (334-450 oocytes per treatment).

Oocytes were stained as described by Cherr et al. (1988). Briefly, cumulus cells were removed with 0.2% hyaluronidase (Hyalozima™, Aspen, São Paulo, Brazil), and the zona pellucida was removed with 0.5% pronase and acid solution (pH 2.5). Oocytes were then fixed in 3% formaldehyde (Mallinckrodt, Paris, KY, USA) and incubated in blocking solution (PBS containing 1 mg/mL BSA, 100 mM glycine [Plusone, La Jolla, CA, USA], and 0.2% sodium azide), left to sit overnight at 4 ºC. For permeabilization, oocytes were treated with 0.1% Triton X-100 (USB, Cleveland, OH, USA) and incubated in 10 µg/mL fluorescein isothiocyanate-conjugated *Lens culinaris* agglutinin and 10 µg/mL Hoechst 33342. Oocytes were then mounted on slides and examined under an Olympus IX-FLA-70 epifluorescence microscope (Tokyo, Japan) to evaluate meiotic progression (excitation of 330-385 nm and emission of 420-490 nm) and the distribution of the cortical granules (excitation of 460-490 nm and emission of 515 nm). Images of each oocyte were captured with an AxioCam camera and stored using AxioVision 4.7.1 software (Carl Zeiss, Jena, Germany). MII oocytes exhibiting cortical granules homogenously distributed at the periphery were defined as matured according to the classification of Hosoe & Shioya (1997).

IVF was performed 24 h after IVM in mineral oil-covered droplets of 100-µL TALP-IVF medium (TALP medium supplemented with 30 µg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine and 1.8 µM epinephrine) containing 0.2 mM pyruvate and 83.4 µg/mL amikacin and supplemented with 6 mg/mL BSA or OVA.

Frozen semen straws (0.5 mL) were thawed in water at 35 ºC for 30 s and prepared by centrifugation on a 45% to 90% Percoll gradient at 900 x *g* for 30 min in TALP-semen medium (TALP medium supplemented with 10 mM acid HEPES [J.T. Baker, Phillipsburg, NJ, USA]). A 30-µL aliquot was taken for evaluation of sperm concentration and motility. Final concentration was adjusted to 25×10^6 motile spermatozoa/mL with TALP-IVF medium. Next, 8 µL of diluted semen were added to the droplets containing 20 to 25 oocytes, for a final concentration of 2×10^5 motile spermatozoa/droplet (2×10^6/mL), corresponding to 8-10 $\times 10^3$ spermatozoa per oocyte. Ooocytes were co-incubated with the sperm for 18 to 20 h at 38.5 ºC in an atmosphere of 5% CO_2 in air under saturated humidity. The semen from the same bull was used for all treatments.

Eighteen to 20 h after the beginning of IVF, the cumulus cells were removed from the presumptive zygotes by successive pipetting. Zygotes were stained with 10 µg/mL Hoechst 33342 for 10 min, and the presence and number of pronuclei were evaluated under an epifluorescence microscope (excitation of 330-385 nm and emission of 420-490 nm). For this purpose, 508 presumptive zygotes were divided into groups, according to experimental design, and analyzed in quadruplicate (79-90 zygotes per treatment).

After IVF, presumptive zygotes were cultured in 4-well dishes containing 500 µL of synthetic oviductal fluid (SOF) supplemented with 10% FCS, 4 mg/mL BSA, or 4 mg/mL OVA under mineral oil. Dishes were incubated in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 38.5 °C in a modular chamber (Billups-Rothenberg, Del Mar, CA, USA) under saturated humidity for 7 to 9 days.

Cleavage rate was determined approximately 32 to 36 h post-insemination under a stereomicroscope (magnification of 50 X). Cleavage was defined as the presence of two-to-four-cell embryos. Development toward blastocyst stage was evaluated on day 7 after IVF. Hatching was evaluated on day 8 after IVF. Blastocyst rate was calculated based on number of treated oocytes, and hatching rate was based on number of blastocysts. For this purpose, 2,355 bovine oocytes were divided into six experimental groups and analyzed in five replicates (314-352 oocytes per treatment).

The morphological quality of the blastocysts produced *in vitro* was evaluated by the proportion of total cells allocated to the inner cell mass (ICM) and trophectoderm (TE). For this purpose, 390 blastocysts derived from six experimental groups were analyzed in triplicate (52-59 blastocysts per treatment).

Blastocysts were stained according to the technique of Iwasaki et al. (1990). Briefly, the zona pellucida of the blastocysts obtained on day 7 was removed by incubation in 0.5% pronase, followed by incubation in acid solution (pH 2.5). Next, blastocysts were washed in medium without FCS and incubated on ice in 10 mM picric acid (Reagen™, Rio de Janeiro, Brazil) and 3 mg/mL PVP. Blastocysts were then incubated in rabbit anti-bovine serum (1:10) at 38.5 °C for 30 min, followed by incubation in guinea pig complement (1:10) containing 10 µg/mL propidium iodide and 10 µg/mL Hoechst 33342 at 38.5 °C for an additional 30 min. Blastocysts were placed on glass slides, covered with glass coverslips and examined under an epifluorescence microscope (excitation of 340-380 nm and emission of 430 nm) to count the number of ICM cells, whose nuclei were stained blue with Hoechst 33342, and TE cells, whose nuclei stained pink with propidium iodide and Hoechst (Koo et al., 2002).

In a preliminary experiment, three concentrations of OVA were used for IVM. Effects were evaluated by means of nuclear maturation and cortical granule migration. IVM medium was supplemented with 2, 4 or 6 mg/mL OVA. Data from three replicates were used, for a total of 676 oocytes.

In order to determine the effects of supplementation by different protein sources during the three steps of *in vitro* embryo production (IVM, IVF and IVC), an experimental design was performed. Combinations of the same supplement for IVM+IVC were combined with BSA or OVA for IVF.

The effect of the source of protein supplementation on IVM was assessed by supplementing the IVM medium with 10% FCS, 4 mg/mL of BSA or 4 mg/mL of OVA. Oocytes were evaluated for nuclear maturation and cortical granule migration in quadruplicate, for a total of 1,168 oocytes.

The effect of treatment on pronuclear formation during IVF was evaluated using a factorial design, which consisted

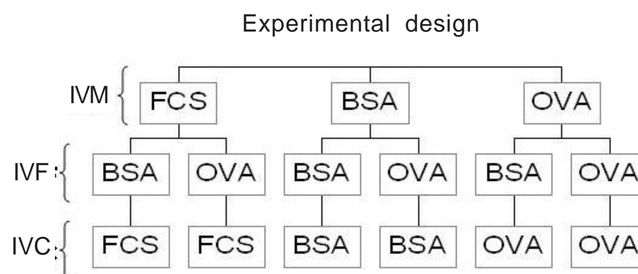


Figure 1 - Schematic of the experimental design used to evaluate the performance of OVA compared with BSA and FCS in IVM+IVC and OVA compared with BSA for IVF of bovine oocytes.

of three sources of supplementation during IVM (FCS, BSA and OVA) and two sources of supplementation during IVF (BSA and OVA). Three replicates per group were performed, for a total of 508 oocytes.

For the IVC step, embryo development (cleavage, blastocyst and hatching rates) and cell number (ICM, TE and total number) results were determined and analyzed by a factorial design consisting of three sources of combined supplementation during IVM and IVC (FCS, BSA and OVA) and two sources of supplementation during IVF (BSA and OVA), for a total of six experimental groups. For IVC, five replicates per group were performed, for a total of 2,003 oocytes. To determine embryo cell number, 324 embryos were stained (51 to 59 embryos per group).

Nuclear maturation and cortical granule migration results were analyzed by ANOVA. Pronuclear formation, embryo development rates (cleavage, blastocyst and hatching rates), embryo cell numbers, and their interaction were analyzed by ANOVA. Means were compared by the Tukey test. The equality of variance was tested by Levene's test. Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Inc., 1989). A level of significance of 5% was used for all experiments.

Results and Discussion

Appropriate *in vitro* bovine embryo production (IVP) systems with high sanitary standards are fundamental in commercial bovine IVP settings, and they depend largely on FCS and BSA's substitution. Especially due to the preponderance of Nellore in its herd, Brazil has developed an expertise in bovine biotechnology and has successfully applied it towards large-scale *in vitro* bovine embryo production (Pontes et al. 2009, 2010). For the first time, the introduction of OVA was proposedas a supplement for bovine embryo IVP to improve the sanitary quality of

bovine embryos produced *in vitro*. The adequate concentration and the effects of OVA in the three main steps (IVM, IVF and IVC) of bovine embryo IVP were evaluated by measuring the formation of pronuclei and embryo development rates and embryo cell staining.

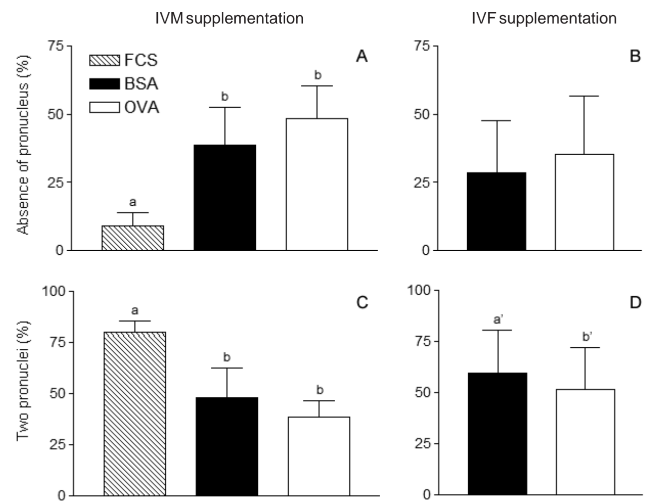
Different concentrations of ovalbumin (2, 4 or 6 mg/mL) for the IVM of bovine oocytes were evaluated by assessing nuclear maturation and cortical granule migration (Table 1). Similar results were obtained with 4 and 6 mg/mL of ovalbumin. Four mg/mL for IVM and for IVC, which are the same amounts used for BSA, were preferred.

In the next experiment, IVM was performed using different protein sources (FCS, BSA, OVA), and nuclear maturation and cortical granule migration were evaluated (Table 2). For FCS, BSA and OVA, similar rates (P>0.05) of nuclear maturation (74.01 to 78.78%) and cortical granule migration (58.89 to 65.48%; Table 2) were observed. Therefore, we can conclude that OVA, FCS, and BSA supplementation enables similar nuclear and cytoplasmic maturation.

To observe the effect of protein source during IVM and IVF, the rate of pronucleus formation and the number of pronuclei formed were evaluated 18 to 20 h post-insemination. No statistical interaction (P>0.05) between IVM and IVF was observed when different protein sources were compared. However, higher rates (P<0.05) of two pronuclei were obtained when FCS was used for IVM (79.97%) compared with BSA (48.06%) and OVA (38.35%) and when BSA was used for IVF (59.4%) compared to OVA (51.52%).

As for the pronuclear formation rates, no interaction (P>0.05) between IVM+IVC and IVF occurred. Data were then statistically compared using protein supplementation for IVM+IVC (FCS [n = 654], BSA [n = 684] and OVA [n = 665]) and protein supplementation for IVF (BSA [n = 1006] and OVA [n = 997]).

Cleavage rates were similar (P>0.05) between treatments when FCS (78.53%), BSA (75.64%) or OVA (69.19%) was used for IVM+IVF and BSA (76.72%) or OVA (72.18%) was used for IVF. IVM+IVC supplementation with BSA or FCS resulted in similar blastocyst rate. Nonetheless, OVA supplementation during IVM+IVC resulted in lower (P<0.05)



Different letters (a, b and a', b') above the bars indicate significant differences (P<0.05) between IVM and IVF supplementation, respectively. Bars depict means, and whiskers depict standard deviations.

Figure 2 - Rates of the absence of pronuclei (A and B) or the presence of two pronuclei (C and D) in zygotes produced from oocytes matured in medium supplemented with fetal calf serum (FCS), bovine serum albumin (BSA) or ovalbumin (OVA) (A and C) or fertilized in medium supplemented with BSA or OVA (B and D).

Table 1 - Nuclear maturation and cortical granule migration after *in vitro* maturation (IVM) of oocytes for 24 h in medium supplemented with 2, 4 or 6 mg/ml ovalbumin

Ovalbumin (mg/mL)	N	Nuclear maturation (% ± SD)	Cortical granule migration (% ± SD)
2	159	50.83 ± 8.42b	20.15 ± 2.31b
4	167	82.66 ± 0.63a	54.21 ± 5.04a
6	170	77.05 ± 5.82a	47.65 ± 4.16a
Total	496	-	-

N = number of oocytes; SD = standard deviation.
a,b = different superscripts within columns indicate differences (P<0.05) between ovalbumin concentrations during IVM.

Table 2 - Nuclear maturation and cortical granule migration of oocytes matured *in vitro* for 24 h in medium supplemented with 10% fetal calf serum (FCS), 4 mg/mL bovine serum albumin (BSA) or 4 mg/mL ovalbumin (OVA)

IVM supplement	N	Nuclear maturation (% ± SD)	CG migration (% ± SD)
FCS	384	75.66 ± 2.88	64.35 ± 5.56
BSA	450	74.01 ± 4.41	58.89 ± 2.88
OVA	334	78.78 ± 4.32	65.48 ± 11.50
Total	1168	-	-

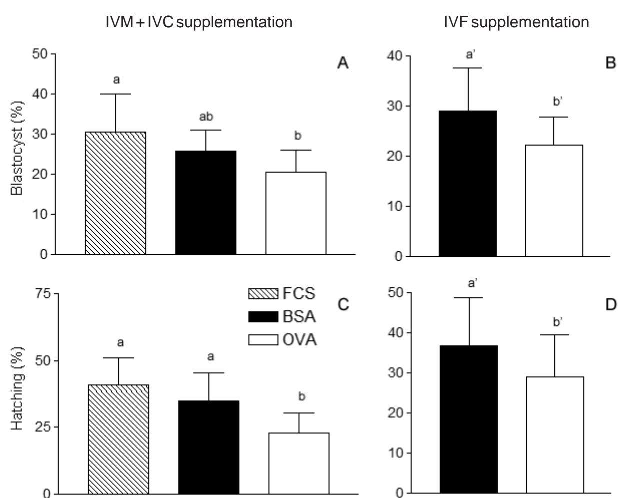
N = number of oocytes; SD = standard deviation; CG = cortical granules. No significant differences (P>0.05) were observed between *in vitro* maturation supplements.

blastocyst (20.40%) and hatching (23.02%) rates than FCS (30.47 and 40.78% for blastocyst and hatching rates, respectively) and BSA (25.73 and 34.82% for blastocyst and hatching rates, respectively) (Figure 3).

Furthermore, the use of OVA during IVF resulted in lower blastocyst development and hatching rates (22.15% and 28.93%, respectively) than BSA (28.91 and 36.82% for blastocyst and hatching rates, respectively).

Because there was interaction between IVM+IVC and IVF supplementation, the six experimental groups were compared using the supplement associations (Table 3). When OVA was used as the supplement for IVF, the use of FCS or BSA as supplements during IVM and IVC resulted in higher (P<0.05) ICM, TE and total cell number compared with OVA for IVM+IVC. When OVA was used as the supplement for IVM+IVC, the use of BSA for IVF resulted in higher (P<0.05) ICM, TE and total cell number compared with OVA as a supplement for IVF. Therefore, supplementation with OVA during IVM+IVC and IVF decreased embryo quality, as measured by the number of ICM, TE and total cells in bovine blastocysts.

The different *in vitro* culture systems that support the development of embryos can be classified according to the presence or absence of protein sources. Media supplemented with FCS are considered undefined, media supplemented with BSA are classified as semi-defined, and media with synthetic supplements are considered defined in their composition. Although defined media would be the ideal system for the *in vitro* culture of oocytes and embryos, supplementation of culture media with protein sources of animal origin provides the best results from oocyte maturation to the final step of *in vitro* embryo development (Vanroose et al., 2001). Synthetic supplements, such as PVA, PVP and Ficoll, can successfully replace serum during IVM when IVC is performed using 5 mg/mL of BSA and 2.5% FCS (Mingoti et al., 2009). Synthetic macromolecules such as polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) have been proposed as substitutes for animal proteins in embryo culture media, but lower embryo development has been observed (Lim et al., 2007).



Different letters (a, b and a', b') above the bars indicate significant differences (P<0.05) among the supplements used for IVM+IVC and for IVF, respectively. Bars depict means, and whiskers depict standard deviations.

Figure 3 - Effect of fetal calf serum (FCS), bovine serum albumin (BSA) or ovalbumin (OVA) supplementation in IVM plus IVC medium or in IVF medium on blastocyst (A and B) and hatching (C and D) rates.

Recently, the use of plant protein hydrolysates or plant peptones (consisting of oligopeptides and free amino acids from wheat and cotton) has been evaluated. Even though similar rates of embryo development were observed, the embryo sex ratio was skewed towards male embryos and needs to be clarified (George et al., 2009). In this study, the aim was to avoid cross-contamination caused by supplements of bovine origin and evaluate the possibility of using OVA, a well-characterized protein supplement of animal origin but with no sanitary risks.

Maturation is complete when the oocyte can be fertilized and develop into an embryo (Rizos et al., 2003). The analysis of nuclear maturation and cortical granule migration (a sign of cytoplasmic maturation) found no significant differences (P>0.05) between the different protein sources (FCS, BSA, and OVA). The rate of nuclear maturation ranged from 74.01 to 78.78% in the different groups, and these rates were similar to the ones reported by other investigators (approximately 80%) for the IVM of oocytes in an undefined medium (Kim et al., 1997).

The success of IVF in cattle depends on the appropriate preparation of both semen and oocytes that favors the metabolic activity of male and female gametes (Brackett et al. 1981). Regarding the rate of pronucleus formation and the number of pronuclei formed, significant differences were observed in the proportion of the absence of a pronucleus and the development of two pronuclei. The capacity of a zygote to form pronuclei depends on the quality of oocyte maturation, and, in this study, FCS was the most efficient protein source for IVM, compared with BSA and OVA. Furthermore, the rate of two pronuclei when oocytes were subjected to IVF with BSA was higher than with OVA, regardless of the supplement used for IVM (FCS, BSA or OVA).

Variations in embryo development results are observed among laboratories due to differences in the procedures, protocols and substances (Gordon, 1994). In the present experiment, no significant (P>0.05) differences in cleavage

rates were observed among treatments. Similar cleavage rates were reported by Pinyopummintr & Bavister (1994), who compared semi-defined or undefined culture media with chemically defined media.

In the literature, the results obtained with BSA and FCS are controversial. The absence of BSA in SOF during IVM increased morulae and blastocyst production (Ali & Sirard 2002), while the presence of BSA-V and FCS during IVC reduced the percentage of morulae and blastocysts, compared with the use of purified BSA, fatty acid-free (FAF) BSA and ovalbumin (Ali & Sirard, 2002). Similarly, Carolan et al. (1995) reported that the removal of BSA from the SOF medium during IVC results in significantly lower blastocyst yields on days 6, 7 and 8. In contrast, Lonergan et al. (1998) observed that FCS significantly accelerated embryo development, triggering premature blastulation and improving blastocyst quality, as demonstrated by an increased total cell number and hatching rate. In media containing FCS, hatching is facilitated by the presence of plasminogen, which assists in the degradation of the zona pellucida (Kaaekuahiwi & Menino, 1990). However, prolonged supplementation with FCS in the culture medium leads to morphological alterations and has deleterious effects on the embryo, affecting cell density, color, size, metabolism and the expression of numerous genes (Krisher et al., 1999; Shamsuddin & Rodriguez-Martinez, 1994; Hall, 1999). Additional harmful alterations include ultrastructural rupture of organelles, such as mitochondria, among others (Hasler, 2000; Farin et al. 2001). Metabolic alterations, such as an abnormally large number of lipid droplets in morulae and blastocysts, have also been reported for embryos cultured in FCS-supplemented medium compared with serum-free medium (Abe et al., 1999).

One of the most practical and efficient approaches to evaluate the embryo quality of blastocysts produced *in vitro* is the quantification of the total number of cells in the embryo and the proportion of cells allocated into the ICM and TE. Bovine embryos of superior quality should have a total cell number as close as possible to the number of cycles. Under the conditions of the present experiment, this number would range from 64 to 128 cells. Total cell number ranged from 56.04 to 84.86 cells per embryo for the treatments using different protein supplement sources. Given the interval of analysis, this mean was distinctly lower than what was expected for the chronological age of the blastocysts.

The use of FCS and BSA during IVM+IVC was efficient for *in vitro* embryo production, regardless of the protein source used during IVF (BSA or OVA). However, OVA supplementation during all three steps (IVM, IVF and IVC) resulted in embryos with a smaller total number of cells (<64 cells, corresponding to the sixth cell cycle), which were classified as low quality. Similar lower embryo quality was observed when FCS and BSA were replacedby PVA during IVC. A mean total number of 81 cells for blastocysts was produced in medium containing FCS, compared with 156 cells for medium supplemented with BSA and 76 cells for medium containing PVA (Kuran et al., 2001).

Conclusions

The present results show that it is possible to produce bovine embryos in the absence of FCS or BSA using OVA as a protein source. Although the quantity and quality of bovine blastocysts using only OVA throughout IVP were lower than those obtained in the presence of FCS and BSA, OVA used during IVF and IVC was able to maintain blastocyst rate, blastocyst development, and hatching rates when FCS was used during IVM. Further studies combining OVA with growth factors, vitamins and hormones during bovine IVP should be performed to improve culture conditions and define efficient systems for bovine embryo IVP.

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