

Substituição parcial do soro fetal bovino durante cultivo *in vitro* reduz a concentração de fosfolípidios em embriões bovinos produzidos *in vitro*

Partial replacement of fetal bovine serum during in vitro culture decreases phospholipid content in in vitro produced bovine embryos

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Resumo

Soro fetal bovino (SFB) e albumina sérica bovina (BSA) são componentes importantes do cultivo *in vitro* (CIV) de embriões bovinos, porém são frequentemente associados ao acúmulo excessivo de lipídios, podendo prejudicar o desenvolvimento embrionário. Este estudo teve como objetivo substituir parcialmente o SFB por BSA V FAF durante o CIV de embriões bovinos, avaliar a produção embrionária e quantificar os lipídios dos embriões, SFB e dos meios de cultivo. Para isto, os embriões desenvolveram em meios de cultivo suplementados com 10% de SFB (SFB10%) ou 5% de SFB e 0.03g de BSA V FAF (SFB5%/BSA). O conteúdo lipídico foi avaliado por UHPLC-MS/MS. A análise estatística foi feita utilizando teste t e ANOVA. A substituição parcial de SFB por BSA V FAF não alterou a produção embrionária. Nos dois grupos foram identificados 10 fosfolípidios e três deles, DOPC (p=0,037), POPG (p=0,046) e C24: 1-SM (p=0,009), apresentaram menores concentrações no meio SFB5%/BSA. Os fosfolípidios identificados nos embriões coincidem com os encontrados no SFB e meios de cultivo e quatro deles DOPC (p=0,013), DPPC (p=0,004), POPG (p=0,05) e C24:1-SM (p=0,003) diminuíram a concentração com a redução do SFB. A substituição parcial do SFB diminuiu a concentração de fosfolípidios sem prejudicar a produção embrionária, sugerindo uma melhora nas técnicas relacionadas ao cultivo *in vitro*.

Palavras-chave: Embriões bovinos; Produção *in vitro* (PIV); Fosfolípidios; UHPLC-MS/MS

Abstract

Fetal bovine serum (FBS) and bovine serum albumin (BSA) are important components during bovine embryo in vitro culture (IVC), but they are associated with excess of embryonic lipid, which might impair embryo development. This study aimed to partially replace FBS by BSA V FAF during bovine IVC, evaluate embryo production and quantify the phospholipid content in produced embryos, SFB and IVC medium. The embryos were in vitro cultured in medium supplied with 10% of FBS (FBS10%) or with 5% of FBS plus 0.03 g BSA V FAF (FBS5%/BSA). The lipid content was evaluated using UHPLC-MS/MS and statistical analysis was performed using t-test and ANOVA. The partial replacement of FBS by BSA V FAF did not alter embryo production. Ten phospholipids were identified in both groups and three of them, DOPC (p=0.037), POPG (p=0.046) and C24: 1-SM (p=0.009) presented lower concentration in FBS5%/BSA culture medium. The phospholipids identified on embryos matches with those found on SFB and culture medium and four of them DOPC (p=0.013), DPPC (p=0.004), POPG (p=0.05) and C24:1-SM (p=0.003) reduced its concentration when FBS was reduced. These findings shown that the FBS partial replacement reduces phospholipids content in embryos but do not decrease embryo production, suggesting a technical improvement.

Keywords: Bovine embryo; In vitro production (IVP); Phospholipids; UHPLC-MS/MS

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Introduction

Assisted reproductive technologies (ARTs) are frequently used in domestic animals and a high number of bovine embryos are produced annually, but these techniques still reduce the quality and embryo rate production (Cagnone and Sirard, 2014). The development of a zygote until blastocyst stage during *in vitro* culture (IVC) is considered the most critical period of *in vitro* production (IVP) of embryos (Lonergan et al., 2003). This stage requires exogenous components such as fetal bovine serum (FBS) and bovine serum albumin (BSA) that stimulate cell proliferation and differentiation (Varago et al., 2008).

Although FBS is considered important to embryo development, it has been shown that its presence on IVC increased 30% of embryo lipid concentration on day 6 of development (Leroy et al., 2005). The FBS may influence the embryo lipids uptake by changing the mitochondria metabolism (Abe et al. 2002; Pereira e Marques, 2008) leading to an abnormal accumulation of long chain saturated and unsaturated lipids in the cytoplasm, which may impair the embryonic development (Abe et al., 2004). Furthermore, the presence of FBS during IVC could alter the concentration, function and chemical property of phospholipids that are important to embryo development, with an essential role in the membrane fluidity and permeability (Edidin, 2003; Pitangui-Molina et al., 2017).

It has been shown that *in vitro* produced embryos seem to be more sensitive to cryopreservation when compared with embryos *in vivo* produced, due to the higher concentration of lipid droplets found in *in vitro* embryos (Sanches et al., 2013). The components captured by the embryo during IVC seems to influence its quality. Small changes in the IVC medium can significantly increase the quality of the produced embryos and it has already been shown that lower lipids content is beneficial for embryo survival, facilitating its storage, transport and trade (Varago et al., 2008). Based on FBS published data (Lonergan et al., 2003; Rizos et al., 2003; Abe et al., 2004; Gómez et al., 2008), we hypothesize that partial replacement of FBS during embryo culture reduces lipid content without impair embryo production and that FBS may be the phospholipid source to embryo during IVC. Therefore, this paper aimed to partially replace FBS by BSA V FAF during IVC and evaluate embryo production and phospholipid concentration on embryos, FBS and both IVC medium.

Material and Methods

In vitro production of bovine embryos

All experiments were performed on Medical School of Ribeirão Preto, SP (FMRP-USP), Brazil. The ovaries were collected from slaughtered cows. Follicles between 2-8 mm were aspirated using an 18-gauge needle connected to a 10 mL syringe. The oocytes were classified according to Leibfried and First (1979) and only those with more than three cumulus cells layers and homogenous ooplasm, grade I (GI) oocytes, were selected. The experiments were carried out in six replicates and a total of 946 cumulus oocytes complex (COCs) were selected. The oocytes were placed in microdrops (20 oocytes/microdrop) of 100 μ L of *in vitro* maturation (IVM) medium [TCM199 with Earle's salts, glutamine, NaHCO₃ pyruvate (22 μ g/mL), 10% of FBS, FSH (0.5 mg/mL), LH (50 μ g/mL), amikacin (83 μ g/mL), and estradiol (1.0 μ g/mL)], during 22-24 hours, in maximum humidity, at 38.8 °C and 5% CO₂.

At the end of IVM, the oocytes were transferred to *in vitro* fertilization (IVF) medium (TALP supplemented with heparin (10 mg/mL), pyruvate (22 μ g/mL), BSA FAF (Fatty acid free) (6 μ g/mL), and PHE solution (2 μ M of penicillamine, 1 μ M of hypotaurine, and 0.25 μ M of epinephrine) plus amikacin (83 μ g/mL) with the sperm to IVF. Each drop contained approximately two million sperm per milliliter (2×10^6 sperm/mL). Gametes stayed on IVF medium during 12 hours in maximum humidity, at 38.8 °C and 5% CO₂.

Twelve hours post-insemination, the presumptive zygotes were removed from the IVF medium, divided into two groups, (1) FBS10% and (2) FBS5%/BSA, and transferred to *in vitro* culture (IVC) medium (day zero, D0). The FBS10% group developed in CR2 stock culture medium (Rosenkrans et al., 1993) supplemented with 10% of FBS, 0.03g of BSA V (Fraction V), 100 μ L of alanine, 100 μ L of glycine and amikacin (83 μ g/mL). The FBS5%/BSA group was culture in the same medium mentioned above with a reduction of 50% of FBS (only 5% of FBS) and addition of 0.03g of BSA V FAF. The



embryos were co-cultured in a cumulus cells monolayer system, without O₂ control, in maximum humidity, at 38.8 °C and 5% CO₂. The embryo production rate was obtained at day 7 of development (D7) based on number of blastocysts in relation to zygotes (D0). Expanded blastocysts were selected to phospholipids analysis, in which 35 embryos from each group were pooled and evaluated in experimental triplicate. Embryos were stored in 100 µL of 0.9% saline solution at -80°C until phospholipid analysis.

Phospholipid analysis

The phospholipid analysis was performed on expanded blastocysts from both FBS10% and FBS5%/BSA groups. To observe the phospholipids concentration in the medium components, 500 µL of FBS alone and the CR2 medium supplemented with FBS10% (CR2-FBS10%) or FBS5%/BSA (CR2-FBS5%/BSA) were evaluated.

Total lipid extraction was made following protocol adapted from Bligh and Dyer (1959). On each sample were added 250 µL of methanol (MeOH) and 125 µL of chloroform, and the solution was sonicated (four times, ten seconds each). Then, 100 µL of water and 125 µL of chloroform were added, shaking using a vortex. The sample was centrifuged for one minute at 1200 x g and two phases could be observed (upper phase: aqueous; lower organic phase: chloroform). The lower organic phase was carefully discarded, 250 µL of chloroform was added to the upper aqueous phase and the sample was centrifuged for one minute (this step was repeat two times in order to extract the maximum amount of material). With the extract separated from the rest of the sample, 300 µL of NaCl was added to the aqueous phase to prevent emulsion formation. The resulting organic layer was exposed to gas nitrogen to dry it. Samples were resuspended in 100 µL of MeOH, and an aliquot of 1 mL was injected to the UHPLC-MS/MS system.

Phospholipid identification by UHPLC-MS/MS

The ultra-high-performance liquid chromatography by mass spectrometry (UHPLC-MS/MS) analysis of phospholipids was performed in an *Acquity Ultra Performance Liquid Chromatographer H-Class* (Waters Corp., Milford, MA, USA) interfaced with the Xevo TQ-S mass spectrometer equipped with an orthogonal Z-spray electrospray ionization source (Waters Corp., Milford, MA, USA). The analyses were detected in the Multiple Reaction Monitoring scan mode (MRM) that is the most specific and accurate method based on the selection of certain precursor ions and their fragments. To optimize the analytical pure standards of phospholipids at 50 ng/mL was diluted in water/acetonitrile (1:1, v/v), ammonium acetate at 10 mM and introduced into the mass spectrometer by infusion at a flow of 10 µL/min and submitted to the fragmentation by CID (Collision Induced Dissociation) with argon. The experimental parameters used were: capillary voltage: 2.30V; source temperature: 150 °C; desolvation temperature: 350 °C; cone gas flow: 150 L/h; desolvation gas flow: 500 L/h; collision gas flow: 0.15 mL; nebulizer gas pressure: 7.00 bar; MRM transition cycle of 3.10⁻³s.

The electrospray ionization source (ESI) operated in both the positive and negative modes. The data obtained were processed in Masslynx 4.1 software (Waters Corporation, Milford, MA, USA) and are shown in supplementary material (Figure S1). The employed method was capable of identify 25 phospholipids classes. The phospholipid standards employed were described on supplementary table S1. For the chromatographic separation of compounds an UPLC BEH columns C4, (Waters Corporation, Milford, MA, USA) with dimensions of 1.7 µm, 50 x 2.1 mm was employed. The elution of compounds was carried out by means of a binary gradient where phase A consisted of water/ammonium acetate 10 mM and phase B was acetonitrile ammonium acetate 10 mM. The elution gradient was varied in the following proportion: 50% B in 0 min, 70% B in 8.0 min, 100% B of 8.5 min to 10.5 min and 50% B from 11.0 until 15.0 min. The flow rate used was 0.5 mL/min. The parameters used for phospholipids analysis on UHPLC-MS/MS chromatograms in the MRM scan mode are shown in Table S2 and Figure S2 (Supplementary material).

The positive identification of a lipid species in the samples was accomplished by matching the MRM transition and retention time of the corresponding phospholipid standard. Comparison of phospholipid levels in the samples was accomplished by analysis of the relative intensities of the analytical signal of the identified phospholipids in the studied samples.



Statistical analysis

Statistical analysis was performed to evaluate the embryo production, phospholipids concentration on embryos cultured with FBS10% and FBS5%/BSA, FBS, CR2-FBS10% and CR2-FBS5%/BSA medium. The mean differences were compared using t-test and One-way ANOVA (Analysis of Variance) with post-hoc Tukey for multiple comparisons. All statistical analyses were carried out using SPSS 22 (IBM®, USA) software with a significance level of 5% ($p < 0.05$).

Results

Embryo production

The *in vitro* production of embryos was not affected ($p = 0.467$) by the partial replacement of FBS by BSA, as the percentage of produced embryos was $46.46\% \pm 7.4$ in the FBS10% group and $43.03\% \pm 5.0$ in the FBS5%/BSA group. Furthermore, the rate of expanded blastocysts was also not affected ($p = 0.924$) by the partial replacement of FBS by BSA (Tab 1).

Tabela 1. Number of selected COCs and embryos cultured in FBS10% and FBS5%/BSA at the first day (D0) and seventh day (D7) of culture ($p < 0.05$).

Group	Selected COCs	Zygotes (D0)	Embryo production (D7) n (% \pm SD)	Expanded blastocysts D7 n (%)*
FBS10%	946	464	207 (46.46 \pm 7.4) ^a	170 (82.1) ^a
FBS5%/BSA		482	206 (43.03 \pm 5.0) ^a	174 (84.4) ^a

Note: Different letters in the same column means statistical difference ($p < 0.05$).

*Expanded blastocyst rates were calculated according to the total of blastocysts at D7.

COCs, cumulus-oocytes complex. FBS, fetal bovine serum. BSA, bovine serum albumin. D0, first day of culture. D7, seventh day of culture. SD, standard deviation.

Phospholipids analyses

A total of ten phospholipids were identified and quantified on expanded blastocysts from both FBS10% and FBS5%/BSA groups (Tab 2). The phospholipids DOPC ($p = 0.037$), POPG ($p = 0.046$), and C24:1-SM ($p = 0.009$) were reduced in embryos cultured with FBS5%/BSA when compared to embryos cultured with FBS10% (Tab 2).

Tabela 2. Phospholipids content (area) in D7 embryos cultured with FBS10% and FBS5%/BSA ($p < 0.05$).

Phospholipids ^A	FBS10% Mean \pm SD (10^6)	FBS5%/BSA Mean \pm SD (10^6)	P value
DMPE	18.5 \pm 6.24	40.9 \pm 3.24	0.305
DOPC	1638.4 \pm 304.8	989.9 \pm 198.0	0.037
DOPE	110.3 \pm 48.1	80.8 \pm 17.8	0.376
DPPC	351.7 \pm 131.6	255.3 \pm 64.6	0.319
POPC	1969.2 \pm 718.5	1395.9 \pm 286.3	0.275
POPE	23.0 \pm 7.55	18.1 \pm 4.63	0.394
POPG	6.50 \pm 1.57	3.07 \pm 1.37	0.046
PS	44.8 \pm 21.0	31.5 \pm 17.2	0.445
C18-SM	127.5 \pm 45.1	79.5 \pm 40.1	0.240
C24: 1-SM	261.9 \pm 22.2	139.1 \pm 39.5	0.009

Note: In bold, $p < 0.05$.

*Phospholipids data were presented based on chromatograms areas.

SD, standard deviation. D7, seventh day of culture.

^ADMPE: dilauroyl phosphatidylethanolamine. DOPC: dioleoyl phosphatidylcholine. DOPE: dioleoyl phosphatidylethanolamine. DPPC: dipalmitoyl phosphatidylcholine. POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. POPE: 1-palmitoyl-2-oleoyl-sn-3-phosphoethanolamine. POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]. PS: phosphatidylserine. C18-SM: N-(octadecanoyl)-sphinganine-1-phosphocholin. C24: 1-SM: N-(15Z-tetracosenoyl)-sphing-4-enine-1 phosphocholine



To evaluate the hypothesis that the phospholipids presented on embryos are also presented in the IVC medium, the FBS alone and both CR2-FBS10% and CR2-FBS5%/BSA medium were analyzed in the UHPLC-MS/MS. The same ten phospholipids found on embryos were found on FBS, CR2-FBS10% and CR2-FBS5%/BSA. Four of them, DOPC ($p=0.013$), DPPC ($p=0.004$), POPG ($p=0.05$) and C24:1-SM ($p=0.003$) showed reduced concentration in CR2-FBS5%/BSA in relation to CR2-FBS10% (Tab 3). It is important to observe that three phospholipids with reduced concentration (DOPC, POPG and C24:1-SM) on CR2-FBS5%/BSA medium also presented lower concentration in embryos cultured in this medium.

Tabela 3. Phospholipids content (area) found on FBS singly, FBS10% CR2 and FBS5%/BSA CR2 medium. ($p<0.05$).

Phospholipids ^A	FBS	CR2-FBS10%	CR2-FBS5%/BSA
	Mean \pm SD (10 ⁶)	Mean \pm SD (10 ⁶)	Mean \pm SD (10 ⁶)
DMPE	12024.89 \pm 4874.78 ^a	9029.57 \pm 2562.70 ^a	11269.77 \pm 1583.92 ^a
DOPC	12163.76 \pm 396.74 ^a	3821.95 \pm 211.03 ^b	2775.38 \pm 280.67 ^c
DOPE	42.59 \pm 3.75 ^a	46.93 \pm 57.72 ^a	7.71 \pm 1.36 ^a
DPPC	7063.87 \pm 144.57 ^a	1568.63 \pm 18.02 ^b	1005.19 \pm 166.88 ^c
POPC	8.99 \pm 1.77 ^a	1.36 \pm 0.36 ^b	0.45 \pm 0.40 ^b
POPE	44.35 \pm 1.44 ^a	19.24 \pm 15.48 ^b	5.02 \pm 1.02 ^b
POPG	76.16 \pm 2.54 ^a	25.55 \pm 9.70 ^b	10.69 \pm 3.94 ^c
PS	26.70 \pm 2.48 ^a	5.18 \pm 0.36 ^b	1.91 \pm 0.18 ^b
C18-SM	11115.91 \pm 796.78 ^a	1676.27 \pm 84.27 ^b	828.50 \pm 149.37 ^b
C24:1-SM	17333.75 \pm 147.05 ^a	5772.75 \pm 728.06 ^b	3321.12 \pm 544.83 ^c

Note: Different letters in the same line means statistical difference ($p<0.05$).

^APhospholipids data were presented based on chromatograms areas.

^ADMPE: dilauroyl phosphatidylethanolamine. DOPC: dioleoyl phosphatidylcholine. DOPE: dioleoyl phosphatidylethanolamine. DPPC: dipalmitoyl phosphatidylcholine. POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. POPE: 1-palmitoyl-2-oleoyl-sn-3-phosphoethanolamine. POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]. PS: phosphatidylserine. C18-SM: N-(octadecanoyl)-sphinganine-1-phosphocholin. C24:1-SM: N-(15Z-tetracosenoyl)-sphing-4-enine-1 phosphocholine. SD, standard deviation.

Discussion

One frequent issue related to the use of FBS during IVP is the embryo lipid amount after *in vitro* culture (IVC). Our results shown that the partial replacement of FBS by BSA V FAF promotes a reduction of the phospholipids DOPC, POPG and C24:1-SM on expanded blastocysts, without impair the production rate. We observed that embryo production on D7 were about 40% in both tested groups what is consistent with the results published in the literature (Viana et al., 2012). The comparison of our results with literature data, shows that partial protein replacement seems to be more adequate than full substitution, since full FBS replacement by BSA during oocyte maturation and embryo culture decreased embryo production (Sena-Netto et al., 2020). One major challenge in IVP is to develop an appropriate growth medium, which mimics the essential components to the proper development of the embryo, reducing cell stress, loss of viability and allowing a technical improvement (Gómez et al., 2008; Cagnone and Sirard, 2014).

FBS and BSA are important components to embryonic early development and are often used during IVC (Crocco et al., 2013). The presence of FBS on IVC stimulates embryo development until blastocyst stage, albeit also influences the lipid content and impair embryo survival after cryopreservation (Rizos et al., 2003; Sudano et al., 2011). Several studies mentioned the relationship between lipid content on embryos and their influence in the success rate of ARTs and other related process. Among them, Sudano and colleagues (2012) showed that membrane phospholipids, for example, can be specie-specific and change its composition according to culture microenvironment, which may result on changes in membrane fluidity and embryo cryosurvival rate. It is clear that lipid metabolism is essential to mammal development (Ferreira et al., 2014) and has fundamental role in energy storage, cell structure and cell physical and metabolic changes (Sudano et al., 2012). Therefore, the quantification and classification of lipids present on embryos are important to understand the embryo metabolism, mainly those produced by



ARTs (Sudano et al., 2012; Melo et al., 2014).

Consistent with published literature (Sudano et al., 2011), our findings demonstrated that a 50% reduction of FBS on IVC did not alter embryo production. Although there was no difference in production rate, the reduction of FBS in the culture medium changed the phospholipids concentration on embryos. The concentrations of DOPC, POPG and C24:1-SM were higher in the FBS10% compared to FBS5%/BSA group. Phospholipids play an important role during and after fertilization, being the major lipid class present in eukaryotic cell membranes (McEvoy et al., 2000; Annes et al., 2019). Preimplantation embryos have higher-mass lipids, such as ceramides, whereas the zona pellucida contains lower-mass lipids such as phosphatidic acid derivatives and phosphatidylethanolamides (Ferreira et al., 2014).

The phospholipids, such as the phosphatidylcholine (DOPC) and sphingomyelin (C24:1-SM) found reduced in the FBS5%/BSA group are structural lipids and membranes components with important role to embryo development, survival rate after cryopreservation techniques and pregnancy success due to its main influence in membrane signaling (Annes et al., 2019). Phosphatidylcholines (DOPC, DPPC and POPC, for example) are the most abundant phospholipids in eukaryotic cell membrane, important to embryogenesis, which may act like a cell messenger and play a role in apoptosis process. Besides that, this phospholipid class is important to endoplasmic reticulum biogenesis, an essential organelle to embryo development (Albi and Viola Magni, 2004; Vance and Vance, 2004; Diez et al., 2005). The sphingomyelins (C18-SM and C24: 1-SM) are structural lipids that participates of cellular signaling and its metabolism products such as ceramides and diacylglycerol, as well as the phosphatidylcholine, may influence cell functions, including cell proliferation and apoptosis (Paula-Lopes and Hansens, 2002; Leão et al., 2014). Furthermore, the sphingomyelins are part of functional membranes and influence membrane permeability and fluidity during temperature changes (Edidin, 2003; Sudano et al., 2012). Still, sphingomyelins could assist nuclear organization during cell division (Albi and Viola Magni, 2004).

To evaluate the hypothesis that the lipids found in bovine embryos are from FBS present in the IVC, we evaluated the phospholipid content of FBS alone and CR2 medium with FBS10% (CR2-FBS10%) and FBS5%/BSA (CR2- FBS5%/BSA). The UHPLC-MS/MS analysis identified the same ten phospholipids found on embryos. Four of them, DOPC, DPPC, POPG and C24:1-SM are phosphatidylcholines, phosphoglycerol and sphingomyelins, respectively, that reduced its concentration on CR2-FBS5%/BSA when compared to CR2-FBS10%. These results are in accordance with Vireque and colleagues (2017) that showed by MALDI-MS method that FBS has elevated phospholipid concentration, specially phosphatidylcholines and sphingomyelins. In addition, three of four phospholipids (DOPC, POPG and C24:1-SM) that presented lower concentrations in CR2-FBS5%/BSA medium were also reduced on embryos that were cultured on it. This corroborate with studies that highlighted the influence of culture medium components during embryo early development such as lipid content (Leroy et al., 2005; Pereira and Marques, 2008).

Despite the importance of the observed results, a limitation of this study was that we did not evaluate if the phospholipid concentration reduction in the FBS5%/BSA group could improve embryo survival rate after cryopreservation. To our knowledge, any study evaluated the association between FBS and phospholipid concentration in embryos cultured in CR2 culture medium with a co-culture system with cumulus cell monolayer and without an O₂ control. Although the studied phospholipids are essential to cell function, structure and organization, their reduction seems not to impair embryo development as observed in production rate results, suggesting that the embryo may capture essential nutrients to its development in other components available in the medium.

In conclusion, the partial replacement of FBS by BSA V FAF reduced the phospholipid content without changing the final embryo production. In addition, the results reinforce the idea that FBS may provide lipids sources to bovine embryos during IVP, since it was identified the same phospholipids on embryos and FBS. Finally, the partial replacement of the FBS reduced the concentration of the same phospholipids on IVC medium and embryos. These results may suggest an improvement of techniques related to IVC, since the reduction of lipid concentration on bovine embryos may increase resistance, improving its survivor and viability.

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