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Use of conjugated linoleic acid (*trans* 10, *cis* 12) to cultivate bovine embryos: effect on cryoresistance and lipid content

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ABSTRACT - This study evaluated the effect of the addition of conjugated linoleic acid (CLA) to *in vitro* culture on viability, lipid content, and cryoresistance of bovine embryos at different *in vitro* culture times. Cumulus oocyte complexes (N = 974) were maturated *in vitro* for 22 h. *In vitro* fecundation ensued for 18 h. Viable zygotes were cultivated *in vitro* in medium supplemented with CLA (100 mM) in the first 72 h (CLA-F), last 72 h (CLA-L), or throughout the culture period (CLA-T). Control embryos (control) were cultivated with no CLA. Embryos were cryopreserved by vitrification for subsequent analysis after devitrification. Effect of CLA on cryoresistance was assessed by cultivating embryos in synthetic oviductal fluid containing 5% fetal bovine serum. Lipid content was quantified using Nile Red staining. No significant difference was observed in cleavage rate, blastocyst:total oocyte ratio, and blastocyst:cleaved oocyte ratio. Culture in CLA-L reduced survival rate 24 h after devitrification compared with CLA-F and CLA-T, although with no statistically significant difference compared with control group. However, CLA-T improved embryo hatching rate and affected lipid content of embryos. Cultures CLA-F and CLA-L increased lipid content compared with control. Yet, lipid content values decreased in embryos treated with CLA-T, but they did not differ significantly from the values observed for oocytes at the germinal vesicle stage. Treatment of bovine embryos with CLA during *in vitro* cultivation did not affect the production of blastocysts, reducing lipid content and improving cryoresistance. However, the effects of CLA on cryoresistance and lipid content is significant only when embryos are exposed to the compound throughout the cultivation period.

Keywords: cryotolerance, polyunsaturated fatty acid, vitrification

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Introduction

In vitro embryos are more sensitive to freezing than *in vivo* embryos and have low hatching rates after devitrification (Sudano et al., 2011). The main reason behind the poor freezability of *in vitro* embryos is the high lipid contents (Rizos et al., 2003; Seidel Jr., 2006; Barceló-Fimbres and Seidel Jr., 2011). Among the main factors identified as responsible for the increase of lipid content in *in vitro*-produced embryos is the addition of fetal calf serum (FCS) in culture media (Seidel Jr., 2006; Lapa et al., 2011; Sudano et al., 2011). Fetal calf serum may contain factors that stimulate the production of lipogenic enzymes (Barceló-Fimbres and Seidel Jr., 2007), and the activity of the lipogenic metabolic pathway may increase due to the high levels of glycose in FCS (Rizos et al., 2003), and this may result in changes in the energy metabolism.

Mammal preimplantation embryos undergoes a marked change in energy metabolism during embryo genome activation. Until the 8- to-16 cell stage, embryos use essentially pyruvate as energy source. After compaction, glycose consumption increases sharply (Khurana and Niemann, 2000). In *in vitro* bovine embryos, the 8- to-16 cell stage starts about 96 h after fertilization, or 72 h into cultivation (Leidenfrost et al., 2011).

This marked change in this specific period has led to the proposition of sequential culture media, in which factors are added or withdrawn according to the moment of embryo development (Wrenzycki, 2016).

To reduce lipid levels and improve cryoresistance of *in vitro* embryos, polyunsaturated fatty acids, like conjugated linoleic acid (CLA; *trans* 10, *cis* 12), have been added to culture media (Pereira et al., 2007; Al Darwich et al., 2010; Stinshoff et al., 2014; Absalón-Medina et al., 2014; Batista et al., 2014; Leão et al., 2015). Conjugated linoleic acid changes the fatty acids profile during maturation, inhibiting the expression of genes that code for lipogenic enzymes and reducing lipid content, while cryoresistance improves, especially when media containing FCS are used (Lee et al., 1998; Baumgard et al., 2002; Pereira et al., 2007; Al Darwich et al., 2010).

Thus, the objective of the present study was to evaluate the effect of the supplementation with CLA at different times of the embryo *in vitro* cultivation (pre- and post-compaction). To this end, embryo production, lipid content, and cryoresistance were analyzed in a production model using bovine fetal serum.

Material and Methods

All stages of the present study were carried out in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil (21°45'16" South, 41°19'28" West).

Embryos were produced according to Pontes et al. (2011) with adaptations. Briefly, *cumulus oophorus* complexes (COC) aspired from ovaries collected from female bovines in a local slaughterhouse were used. The COC were transported to the laboratory in sterile saline (NaCl 0.9%) supplemented with antibiotics (penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}) at approximately 25 °C throughout the journey. The transport time between the slaughterhouse and the laboratory ranged from 1 to 2 h. In the laboratory, ovaries were washed in the same solution with antibiotics used for transportation and were subsequently subjected to follicle puncture.

Ovarian follicles (3-8 mm) were punctured and had their contents removed into a conical tube. The supernatant was decanted, and between 3 and 5 mL of a manipulation medium (TCM supplemented with Earle's salts, HEPES 25 mM, FCS 10%, penicillin 100 IU mL^{-1}, and streptomycin 100 mg mL^{-1}) were added to the precipitate. The mixture was transferred to a Petri dish to search and retrieve COC, which were subsequently inspected and classified. Only COC with more than three compact layers of *cumulus* cells and homogeneous cytoplasm were used.

For *in vitro* maturation (IVM), the selected COC were washed in manipulation medium and then transferred to maturation medium (TCM 199 with Earle salts, with 10% of fetal calf serum, 20 μg mL^{-1} FSH, 100 mg mL^{-1} of streptomycin sulfate, and 100 IU/mL of penicillin). The COC were placed in petri dishes, containing 25-30 oocytes each and covered with mineral oil. The IVM was performed for 22 h in incubator at 38.5 °C in 5% CO_2 with saturated humidity.

At the end of the maturation period, the oocytes were transferred to fertilization media. Oocytes were subjected to *in vitro* fertilization (IVF) with frozen semen from a single sample of a bull with proven fertility. Viable sperms were selected using the Mini-Percoll gradient technique. Briefly, the gradient was prepared transferring 400 μL Percoll 90% to a microcentrifuge tube. On top of that, 400 μL Percoll 45% was poured. Semen was carefully deposited at the top of the gradient in the tube, and the contents were centrifuged at 600 × *g* for 15 min. The supernatant was discarded, and the precipitate was resuspended in 1.0 mL SP-TALP medium. The second centrifugation was run at 600 × *g* for 5 min. The supernatant

was discarded, and the precipitate was resuspended in 100 µL fertilization medium prepared with TALP supplemented with 6 mg mL^{-1} fatty acid-free bovine serum albumin (BSA), 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 2 mM penicillamine, 1 mM hypotaurine, 25 mM epinephrine, and 0.2 mM sodium pyruvate.

Mature COC were washed twice in FERT-TALP medium (Nutricell®) and transferred to 100-µL fertilization microdroplets under mineral oil containing semen diluted to a concentration that generated a 2×10^6 sperms mL^{-1} fertilization dose. *In vitro* fertilization was carried out for 18 h in a CO_2 incubator under the same IVM conditions.

After fertilization, viable zygotes were partly denuded, washed, and cultivated for seven days in synthetic oviductal fluid (SOF) containing FCS 5% (Nutricell®). Then, CLA (100 mM) was added in the first 72 h (CLA-F), last 72 h (CLA-L), and throughout the cultivation period (CLA-T). Control embryos were cultivated with no CLA. *In vitro* cultivation was carried out using 20 COC per 100-µL microdroplet under mineral oil in an incubator at 38.5 °C with 5% CO_2 atmosphere and humidity saturation. Cleavage and blastocyst formation rates were analyzed 48 and 168 h after fertilization, respectively.

Embryos were cryopreserved by vitrification (Vajta et al., 1997). Initially, embryos were placed in maintenance medium (TCM 199 HEPES, FCS 20%) for 1 min. Then, they were transferred to equilibrium solution (SV1) composed of ethylene glycol 10% (EG) and dimethyl sulfoxide 10% (DMSO) diluted in the maintenance medium described, where they remained for 3 min. Next, embryos were placed in the vitrification solution (EG 20%, DMSO 20%) diluted in described maintenance medium for 30 s.

During vitrification, embryos were loaded in open pulled straws (OPS, N = three embryos per straw) and immediately immersed in liquid nitrogen.

Devitrification was carried out by placing the narrow end of the OPS in the devitrification solution (maintenance solution, saccharose 0.3 M). Embryos were removed from the medium and immediately transferred to a well containing the same solution, where they were kept for 5 min. Then, embryos were transferred to yet another well containing a devitrification solution formed by the maintenance solution and saccharose 1.5 M, where they remained for another 5 min. Lastly, embryos were transferred to maintenance solution. All vitrification and devitrification solutions were maintained at 39 °C, and procedures were carried out at the same temperature.

Devitrified embryos were cultivated in commercial SOF containing FCS 5% (Nutricell®) for 48 h. Survival rate was assessed 24 h after the beginning of cultivation. Blastocele reexpansion or hatching were the criteria used to consider an embryo viable. Hatching rate was assessed 48 h into cultivation and included all hatched embryos during the cultivation period.

Lipid contents of embryos were quantified using the Nile Red technique (Batista et al., 2014) with slight modifications. Oocytes and embryos (N = 68, between 10 and 14 per treatment) were fixed in phosphate buffered saline (PBS) containing formalin 10% and polyvinyl alcohol (0.01%). Oocytes and embryos were then transferred to a 500-µL PBS microdroplet containing Nile Red 1 µg mL^{-1} (Sigma N-3013) and kept overnight under these conditions. Next, the material was washed twice in PBS and mounted on a slide with glycerol and topped with a coverslip. All solutions used were stored in a dark environment, and analyses were carried out protected from light. Slides were inspected in an epifluorescence microscope (Eclipse TE 300, Nikon) to induce excitation of the dye and render visible the lipid contents of embryos. Each embryo was photographed using a specific digital camera (Nikon DS Ri1) coupled to the microscope. Images were analyzed using the NIS-AR software version 3.1 (Nikon).

In addition to embryos, oocytes at the germinal vesicle stage (GV, fixed immediately after follicle puncture) and maturated oocytes (MII, 22 h after *in vitro* maturation) were analyzed.

Lipid content of each embryo and of oocytes (GV and MII) treated with CLA at different times of cultivation (CLA-F, CLA-L, CLA-T, and control) were assessed using the specific software platform, according to which the higher the fluorescence, the higher the lipid content of a sample. Image pixels of embryos or oocytes were used to quantify the emission of fluorescence and scored 0 to 255 arbitrary

fluorescence units (AFU). Data were exported to Excel spreadsheets (Microsoft®, 2010) for subsequent statistical analysis.

The data obtained were analyzed in the R environment (version 2.11.1). The statistical model used was:

$$Y_{ij} = \mu + t_i + e_{ij},$$

in which Y_{ij} = value observed in the experimental unit i for repeat j, μ = general mean, t_i = effect of treatment i, and e_{ij} = residual error.

The experiment was carried out seven times (repeats), and each repeat was considered the experimental unit, from which cleavage rate, blastocyst rate, hatching rate, and lipid content were obtained for the different treatments.

Normality of data was assessed using the Kolmogorov-Smirnov test and data that did not fit the normal distribution (lipid content) were analyzed using the non-parametric Kruskal-Wallis test with multiple comparison of means. Cleavage rate, embryo yield, survival rate, and hatching rate data were evaluated using an analysis of variance. Means that differed statistically were analyzed using the Tukey test. The level of significance used in all analyses was 5%.

Results

In total, 974 oocytes were used to assess the effect of CLA at different moments into cultivation. No statistically significant differences (P>0.05) were observed between treatments, whether in cleavage rate, or blastocyst:total oocyte and blastocyct:cleaved oocyte ratios (Table 1).

The fluorescence labeling of lipids carried out using Nile Red showed that CLA-T embryos had the lowest lipid content, with values that were similar to those observed for GV oocytes. In other words, these structures were not affected by the settings of the *in vitro* cultivation medium. Oppositely, embryos cultivated with CLA-F and CLA-L had higher lipid content than control embryos and MII oocytes (Figures 1 and 2).

Cryoresistance was assessed using 246 vitrified embryos that were randomly assigned to treatments. Embryos in CLA-L had a significantly lower (P<0.05) survival rate 24 h after vitrification compared with CLA-F and CLA-T, although with no statistically significant difference compared with control (P>0.05). However, treatment with CLA-T improved hatching rate of embryos (P<0.05) (Table 2).

Discussion

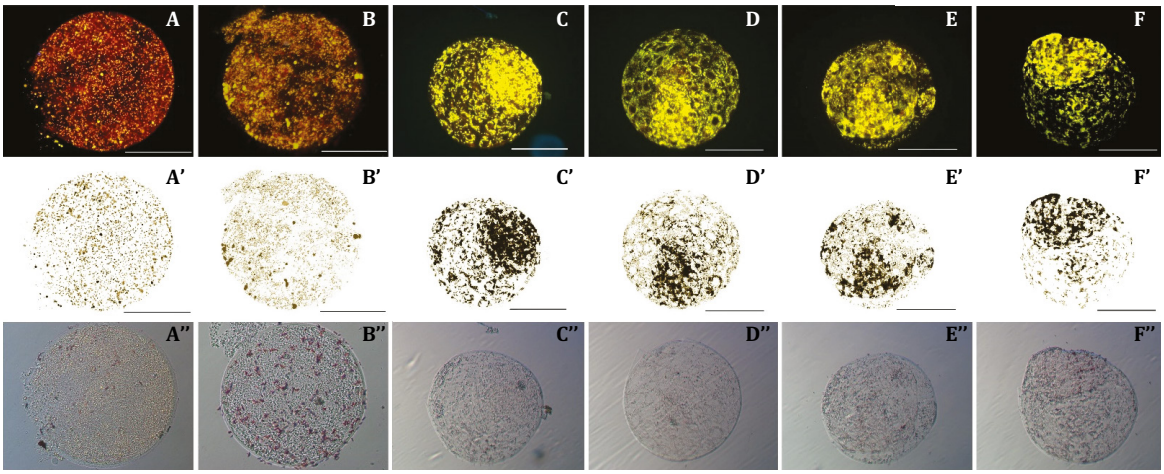
Gardner and Wale (2013) verified that glucose metabolism is abnormal in *in vitro*-produced embryos. According to Bavister (1995), this change is defined as "Crabtree effect", which is characterized by an increase in the metabolism of glucose via glycolysis with inhibition of oxidative phosphorylation. This author also mentions that this effect is not observed in *in vivo*-produced embryos because, possibly in the physiological environment, there would exist a natural glycolysis inhibitor.

Table 1 - Effect of CLA on cleavage rate and blastocyst yield of bovine embryos cultivated *in vitro*

Treatment[1]	Embryo yield			
	Oocytes (n)	Cleavage (% ± SEM)	Blastocysts:oocytes (% ± SEM)	Blastocysts:Cleaved oocyte (% ± SEM)
Control	229	67.6±1.7	33.1±5.0	48.9±7.5
CLA-F	250	62.8±4.9	25.6±3.5	40.0±3.1
CLA-L	247	60.8±4.8	25.9±4.6	43.7±8.8
CLA-T	248	60.7±4.2	32.5±8.3	57.5±16.9

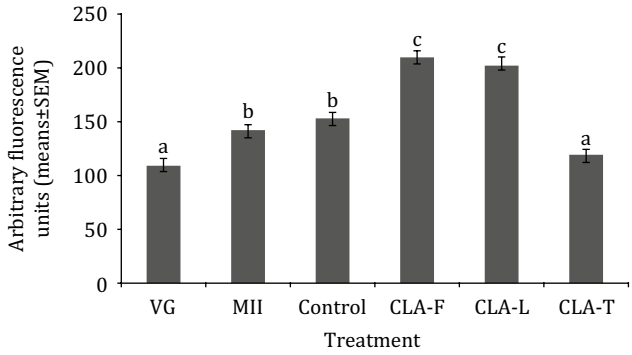
SEM - standard error of the mean.
[1] Control: with no CLA; CLA-F: CLA added in the first 72 h; CLA-L: CLA added in the last 72 h; CLA-T: CLA added throughout cultivation.

The highest activation of the glycolysis pathway, caused by the Crabtree effect, could generate an accumulation of lipids due to the increase in the concentration of fatty acids precursors, such as acetyl-CoA (Rieger et al., 2002). These findings, added to the results of Abe et al. (2002), who observed that the highest lipid content in *in vitro* embryos cultivated in medium with FCS are observed between the 8-cell stage and the morula stage, prompted us to assess the effect of CLA added at different moments into cultivation, namely before and after compaction. In the present study, no statistically



In A to F, the labeling used to quantify lipids based on arbitrary fluorescence units; in A' to F', the inversion of the contrast of the first sequence of images to highlight staining and indicate the location, distribution, and size of lipid microdroplets; in A'' to F'', structures in bright field.
Bars: 50 µm; all images were recorded at 400X magnification.

Figure 1 - Fluorescence labeling by Nile Red of lipid contents of oocytes at the germinal vesicle stage (VG; A), oocytes maturated for 22 h (MII; B), and embryos cultivated with no CLA (control; C) in the first 72 h of cultivation (CLA-F; D), last 72 h of cultivation (CLA-L; E), and throughout cultivation (CLA-T; F).



SEM - standard error of the mean.

Figure 2 - Lipid contents of oocytes at the germinal vesicle stage (VG), oocytes maturated for 22 h (MII), embryos cultivated with no CLA (control), embryos cultivated with CLA in the first 72 h of cultivation (CLA-F), last 72 h of cultivation (CLA-L), and throughout cultivation (CLA-T).

Table 2 - Reexpansion and hatching rates[1] of the embryos cultivated *in vitro*

Treatment[2]	N	Reexpansion (% ± SEM)	Hatching (% ± SEM)
Control	65	50.3±3.5ab	26.0±8.6a
CLA-F	67	60.6±4.6b	33.7±4.3a
CLA-L	64	40.0±8.2a	22.6±3.4a
CLA-T	68	61.8±3.2b	61.8±3.2b

SEM - standard error of the mean.
[1] Reexpansion and hatching rates were measured 24 h and 48 h after devitrification.
[2] Control: with no CLA; CLA-F: CLA added in the first 72 h; CLA-L: CLA added in the last 72 h; CLA-T: CLA added throughout cultivation.

significant difference was observed in embryo yields among treatments. On the other hand, the addition of CLA at different moments during embryo development showed that the compound affects lipid content and cryoresistance.

Embryo cleavage and yield did not differ significantly among treatments (P>0.05; Table 1). These findings show that the CLA concentration used (100 μM) did not have any toxic effect, since it improved cryoresistance with no decrease in embryo yield. Similar findings were obtained by Batista et al. (2014) and Pereira et al. (2007), who also used cultivation media supplemented with FCS and CLA 100 μM. Yet, Stinshoff et al. (2014) used a cultivation medium with no FCS and observed that the addition of CLA during *in vitro* cultivation reduced blastocyst yield. In another study, a decrease in blastocyst yield was recorded when parthenotes were cultivated in a CLA medium with no FCS (Absalón-Medina et al., 2014).

The positive effect of FCS has been demonstrated by various authors, who proved that FCS speeds up the embryo development rate upon the morula stage (Lazzari et al., 2002; Lequarre et al., 2003; Rizos et al., 2003). In addition, FCS has also been shown to increase the number of cells per embryo and induce a rise in percent number of embryos hatched (Holm et al., 2002; Lazzari et al., 2002; George et al., 2008; Sudano et al., 2011). Such advantageous properties of FCS may balance out the harmful effects of CLA on embryo yield as observed by Stinshoff et al. (2014) and Absalón-Medina et al. (2014), making lipid levels in *in vitro* embryos reach similar values as those observed for *in vivo* embryos.

The addition of CLA to *in vitro* culture media at different times of cultivation influenced lipid content of embryos, independently of whether the compound was added in the beginning, end, or throughout the cultivation period. Surprisingly, embryos treated with CLA-F and CLA-L had higher lipid contents compared with the control. However, the embryos treated with CLA-T had lower levels of lipids, compared with the other groups (P<0.05). This may be associated with the change in gene expression pattern of enzymes with a role in lipid metabolism (Lee et al., 1998). It may be hypothesized that the effect of CLA on the downregulation of lipogenic enzymes requires the incorporation of the compound, which means that embryos have to be exposed to CLA for relatively long periods. Or even, that the effect of CLA would be observed only when this component is present during the period of maternal zygote transition, between 72 and 96 h of culture.

Thus, lipid accumulation at levels similar to the control group observed in the CLA-F and CLA-L treatments may have occurred due to the absence of CLA throughout the maternal zygote transition period.

Such results show that CLA needs to be present in the cultivation medium throughout the cultivation process for lipid content to decrease and cryoresistance to increase, which corroborates the idea that CLA must be present throughout the maternal zygote transition period for its effect to be observed. Unfortunately, however, the specific transition period was not assessed in isolation.

In the present study, lipid levels in embryos were similar to the values observed for oocytes that were not subjected to any influence of the *in vitro* conditions (that is, GV oocytes; P>0.05). Recently, Collado et al. (2017) observed that oocytes maturated *in vitro* had significantly high levels of lipids compared with oocytes maturated *in vivo*. The data obtained by the authors suggested that lipid content in oocytes could be the result of the transport of fatty acids produced by COC, which presented changes in the expression of genes that regulate lipid content. This was not observed for oocytes. Besides, these findings may explain, at least in part, the increase in lipid levels in MII oocytes (after *in vitro* maturation) compared with GV oocytes (with no influence of *in vitro* production) (Figure 2).

Baumgard et al. (2002) described the decrease in lipogenic enzyme transcripts in mammary tissue of lactating cows after administration of an intramammary infusion of a CLA solution. The consequence of this reduction in gene expression was low lipid-level milk. Conjugated linoleic acid also affected the expression of enzymes with a role in lipid metabolism in embryos, among which stearoyl-CoA desaturase (SCD1) and fatty acid desaturase 2 (FADS2) (Al Darwich et al., 2010).

Nagashima et al. (1995) used pig embryos to demonstrate, for the first time, that a decrease in lipid levels induces an increase in cryoresistance. After that pioneering study, numerous investigations

have been carried out confirming these results also for bovines (Rizos et al., 2003; Seidel Jr., 2006; Pereira et al., 2007; Pereira et al., 2008; Barceló-Fimbres and Seidel Jr., 2011; Sudano, 2011).

Similarly to the previous results discussed above, the findings obtained in the present study confirm that embryos treated with CLA-T had also the lowest lipid content, showing that CLA may be a useful additive to improve cryoresistance of bovine embryos, mainly in *in vitro* production systems that still use media containing FCS. These results corroborate the findings published by Pereira et al. (2007), who demonstrated, for the first time, that supplementation of embryo culture media with CLA reduces lipid content in embryos and increases cryoresistance.

The beneficial effect of CLA on cryoresistance may also be explained in view of the increase in cytoplasmic membrane fluidity. Leão et al. (2015) observed that CLA changes the lipid profile of the cytoplasmic membrane, a phenomenon that had been previously hypothesized by Hochi et al. (1999). Moreover, CLA is able to change the fatty acid profile, influencing the levels of triglycerides or directly affecting the levels of saturated as well as unsaturated fatty acids, possibly by inhibiting the expression of genes that code for the synthesis of lipogenic enzymes and the activity of lipoprotein lipase. This enzyme hydrolyzes triglycerides into lipoproteins; for this reason, inhibition of lipoprotein lipase increases the intracellular content of triglycerides (Park et al., 1997).

In another study, Al Darwich et al. (2010) evaluated the effect of different polyunsaturated fatty acids on the cultivation of bovine embryos, demonstrating that these fatty acids may reduce levels of FADS2 gene transcripts without affecting the levels of transcripts of other genes associated with lipid metabolism. Downregulation of FADS2 may induce the reduction of lipid content in embryos, since the enzyme catalyzes the biosynthesis of precursors used in the synthesis of polyunsaturated fatty acids.

Conclusions

Treatment of bovine embryos with conjugated linoleic acid during *in vitro* cultivation does not affect the yield of blastocysts, promoting the reduction of lipid levels and improving cryoresistance. Nevertheless, these effects only manifest when embryos are treated with conjugated linoleic acid throughout the cultivation period. Therefore, conjugated linoleic acid may be used as an additive to cultivation media for bovine embryos as a means to increase cryoresistance in production systems that use fetal calf serum.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.P. Carvalho and A.J.B. Dias. Data curation: B.P. Carvalho and D. Detoni. Formal analysis: B.P. Carvalho and F.Q. Costa. Funding acquisition: A.J.B. Dias. Investigation: B.P. Carvalho and F.Q. Costa. Methodology: B.P. Carvalho, F.Q. Costa, D. Detoni, F.B. Rosa and A.J.B. Dias. Project administration: A.J.B. Dias. Visualization: F.Q. Costa. Writing-original draft: B.P. Carvalho and A.J.B. Dias. Writing-review & editing: B.P. Carvalho and A.J.B. Dias.

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