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Lipid characterization of *in vitro*-produced bovine embryos with distinct kinetics of development

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Research Article

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Summary

Human embryo studies have proposed the use of additional morphological evaluations related to the moment of the first cell divisions as relevant to embryo viability. Nevertheless, there are still not enough data available related to morphokinetic analysis and its relationship with lipid composition in embryos. Therefore, the aim of this study was to address the lipid profile of bovine embryos with different developmental kinetics: fast (four or more cells) and slow (two or three cells) at 40 h post-insemination (hpi), at three time points of *in vitro* culture (40, 112 and 186 hpi) and compare these to profiles of *in vivo* embryos. The lipid profiles of embryos were analyzed by matrix-assisted laser desorption ionization mass spectrometry, which mainly detected pools of membrane lipids such as phosphatidylcholine and sphingomyelin. In addition to their structural function, these lipid classes have an important role in cell signalling, particularly regarding events such as stress and pregnancy. Different patterns of lipids in the fast and slow groups were revealed in all the analyzed stages. Also, differences between *in vitro* embryos were more pronounced at 112 hpi, a critical moment due to embryonic genome activation. At the blastocyst stage, *in vitro*-produced embryos, despite the kinetics, had a closer lipid profile when compared with *in vivo* blastocysts. In conclusion, the kinetics of development had a greater effect on the membrane lipid profiles throughout the embryo culture, especially at the 8–16-cell stage. The *in vitro* environment affects lipid composition and may compromise cell signalling and function in blastocysts.

Introduction

Embryo viability and competence have been evaluated by several criteria such as morphology and blastocyst rates (Alikani *et al*., 2002). The advent and application of new biotechnologies have however demonstrated that embryonic competence can be severely compromised without noticeable morphological changes (Vajta *et al*., 2000; Sudano *et al*., 2011; Sugimura *et al*., 2012). Human embryo studies have suggested the use of additional morphological evaluations related to the moment of the first embryonic cell divisions, which appear to be relevant to embryo viability (Lundin *et al*., 2001; Ciray *et al*., 2006; Lemmen *et al*., 2008; Mio and Maeda, 2008; Meseguer *et al*., 2011).

Morphokinetic associations between particular events such as cleavage speed, mainly during early stages of embryo development, have been suggested as predictive markers to improve the selection of embryos for transfer. However this analysis is still considered a controversial method for embryo selection and, apparently, it may vary in different species (Salumets *et al*., 2001; Baumann *et al*., 2007; Meseguer *et al*., 2011; Market-Velker *et al*., 2012; Aparicio *et al*., 2013). In bovine, fast development *in vitro* embryo production (IVP) embryos have been found to display higher blastocyst conversion, lower developmental block and better quality regarding programmed cell death, when compared with slow development embryos, a similar phenotype to those found when antioxidants are added to the individually *in vitro*-cultured (IVC) cells (Garcia *et al*., 2015; Silva *et al*., 2016; Zullo *et al*., 2016; dos Santos *et al*., 2019). At the embryonic genome activation period (8–16 cells), fast development embryos also seem to activate genes related to development, whereas the slow ones activate genes related to cell survival and apoptosis (Ripamonte *et al*., 2012). However, how lipid metabolism occurs in embryos with different speed of development is unknown.

Higher amounts of cytoplasmic lipid droplets and the disturbed composition of membrane lipids of oocytes and IVP embryos are associated with reduced viability and cryosurvival

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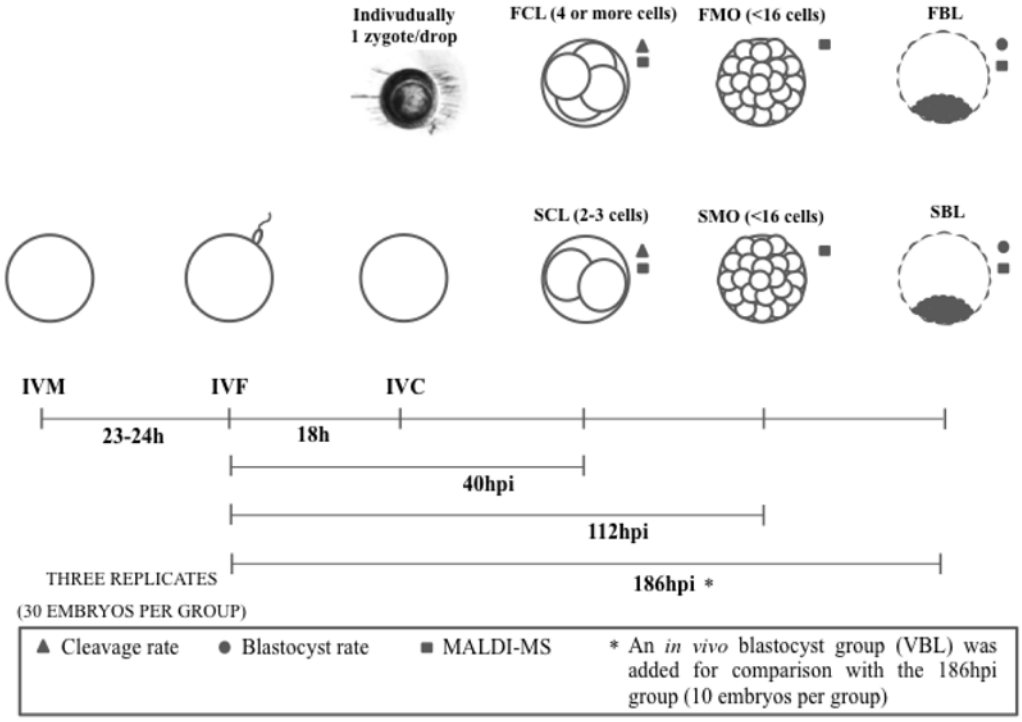

Figure 1. Experimental design. FCL: fast cleavage; SCL: slow cleavage; FMO: fast 8–16 cells; SMO: slow 8–16 cells; FBL: fast blastocyst; SBL: slow blastocyst.

capacity, leading to a higher sensitivity to cryopreservation (Abe *et al.*, 2002; Rizos *et al.*, 2002; Camargo *et al.*, 2011; Sudano *et al.*, 2012; Leão *et al.*, 2014) and conception rates below 30% when compared with their *in vivo* counterparts (Hasler, 2003).

It is known that phospholipids (PL) such as phosphatidylcholine (PC) and sphingomyelin (SM) are major membrane constituents and that their concentration and distribution pattern affect membrane properties, influencing cryosurvival and pregnancy success. These lipids classes are also involved in cell signalling pathways (Ghanem *et al.*, 2014). Over the past years, increasing attention has been given, therefore, to identifying lipid markers of IVP embryos related to their viability and cryotolerance, aiming to improve the success and application of embryo technologies (Sudano *et al.*, 2012, 2013, 2016; Leão *et al.*, 2014). In this work, we describe the lipid profile, with focus on membrane lipids, of *in vitro*-produced bovine embryos derived from fast or slow cleavage cells during early embryo development.

Materials and Methods

Experimental design

Oocytes were *in vitro* matured (IVM) and fertilized (IVF). Presumptive zygotes were IVC, one zygote per drop without sharing the medium (Annes *et al.*, 2017). At 40 h post-insemination (hpi) cleaved embryos were classified as fast cleavage (four cells, FCL) or slow cleavage (two or three cells, SCL) and 30 embryos from each group were frozen in PBS at −80°C for subsequent matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) individual evaluation. Another set of embryos derived from the fast or slow groups was also collected for later MALDI-MS individual analysis at 112 hpi (8–16-cell embryos; fast 8–16 cells (FMO) and slow 8–16 cells (SMO), respectively; $n = 30$), and at 186 hpi (blastocysts; fast blastocyst (FBL) or slow blastocyst (SBL), respectively; $n = 30$) (Fig. 1). As a gold standard, an *in vivo*-derived blastocyst group (VBL) was also analyzed ($n = 10$) for the comparison with FBL and SBL at 186 hpi.

In vitro embryo production

Bovine ovaries were obtained from a commercial slaughterhouse and washed with sterile saline [0.9% (w/v) NaCl containing 100 U/ml penicillin-G and 100 µg/ml streptomycin] at 37°C. Follicles of 2–8 mm in diameter were aspirated with a 21-gauge needle attached to a 5-ml syringe and the fluid allocated in 15-ml conical tubes. Cumulus–oocyte complexes (COCs) were allowed to settle for 10 min and these were selected according to cytoplasm aspect and number of granulosa cell layers.

Groups of 30 COCs were matured in 90-µl droplets of TCM-199 supplemented with 10% fetal bovine serum (FBS), 0.5 µg/ml follicle-stimulating hormone (FSH) (Follitropin-V, Bioniche, Belleville, Canada), 100 IU/ml hCG (Chorulon, Merck Animal Health, Boxmeer, The Netherlands) and 1.0 µg/ml estradiol under mineral oil for 23–24 h at 38.5°C and 5% CO_2 in air with high humidity. *In vitro* fertilization procedures were performed as described earlier (Parrish, 1988). All IVF incubations were performed in an atmosphere of 5% CO_2 in humidified air. Groups of 30 oocytes were inseminated with 1×10^6 Percoll-purified spermatozoa/ml from a pool of three bulls.

After 18 h from the start of fertilization, the remaining granulosa cells were completely removed and each zygote was cultured individually, in a pierced well, in the centre of a 20-µl drop of potassium simplex optimization medium (KSOM) (106-D Millipore) supplemented with 10% FBS, gentamicin and non-essential amino acids, under oil at 38.5°C in a humidified atmosphere with 5% CO_2 without co-culture (Annes *et al.*, 2017). Cleavage rates were assessed after 40 hpi and cleaved embryos were classified into two groups as either fast (four cell) or slow (two or three cell)

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embryos (Milazzotto *et al.*, 2016; Silva *et al.*, 2016). At this time the culture medium was replaced with the synthetic oviduct fluid (SOF) culture medium supplemented with 2% essential amino acids (M-5550, Sigma, USA), 1% non-essential amino acids (M-7145, Sigma, US), and 5% FBS (SOFaa). The blastocyst rates were recorded at 186 hpi. The embryos were classified for quality on the basis of morphological criteria (Robertson and Nelson, 2010) and only grades 1 and 2 embryos were collected for the assay.

Over the three time points both fast and slow groups were collected for analysis (Fig. 1): 40 hpi (FCL and SCL), 112 hpi (FMO and SMO) and 186 hpi (FBL and SBL). An *in vivo*-derived blastocysts group (VBL) was also analyzed for comparison with the IVP blastocyst groups at 186 hpi. In total, 190 embryos were evaluated, 30 embryos per group, except for *in vivo*-produced blastocysts ($n = 10$). Each embryo was washed in PBS and stored at −80°C until MALDI-MS analysis.

In vivo embryo production

In vivo-derived blastocysts were produced in three crossbred cows by a commercial laboratory using previously established methods for oestrus synchronization, superovulation and embryo collection. Briefly, cows had their dominant follicle ablated and received an intravaginal implant of progesterone (day 0). At day 4, the cows started receiving a decreasing dose of FSH (Pluset, Hertape Calier, Juatuba, MG, Brazil) twice a day until day 7 (eight doses from 100 UI to 12.5 IU reaching, in total, 412 IU. A single prostaglandin F2α (PGF2α) (D-cloprostenol, Veteglan, Hertape Calier) injection was administered at the time of the fifth FSH injection. At day 8, donors received a single injection of gonadotrophin-releasing hormone (GnRH) and were artificially inseminated 12 h and 24 h afterwards. At 6.5 days after the last insemination, a non-surgical embryo collection was performed.

MALDI-MS analysis

Equipment calibration was performed using standards of eight peptides with known masses. Samples were prepared as previously described (Sudano *et al.*, 2012; Tata *et al.*, 2013). Briefly, embryos were removed from the culture medium and washed individually in PBS and stored at −80°C until analysis. Each embryo was washed by pipetting in triplicate into MeOH/H_2O (1:1) droplets under a stereomicroscope, spotting them onto the MALDI plate and allowing them to dry at room temperature. The sample location on the spot was annotated using both a stereomicroscope and a sheet of paper reporting a schematic map of the target plate to correctly aim the laser. The matrix was made up by mixing 150 mg of 2,5-dihydroxybenzoic acid in 1 ml of MeOH. Subsequently, 1 µl of the matrix was spotted on each embryo. Mass spectra were acquired in the positive ion mode using an Autoflex III MALDI time-of-flight mass spectrometer equipped with smartbeam™ laser technology from Bruker Daltonics (Bremen, Germany). The MS data were acquired in the range from m/z 700 to 1000 by averaging 1200 consecutive laser shots with a frequency of 200 Hz.

Mass spectra was checked using the FlexAnalysis 3.3 software (Brucker Daltonics). The most abundant ions, after the exclusion of isotopic ion peaks, were considered from each spectrum and used as the starting point to search for ions of m/z values corresponding to lipids (Sudano *et al.*, 2012).

The phospholipid ions detected by MALDI-MS were characterized according to previous studies (Milne *et al.* 2006; Ferreira *et al.*, 2010; Sudano, *et al.*, 2012, 2016; Jung *et al.*, 2014; Shrestha *et al.*, 2014), and by the use of two lipid databases (http://lipidsearch.jp and http://www.lipidmaps.org).

Statistical analysis

Cleavage rates (fast, slow and total) were calculated based on the total number of oocytes. Blastocyst rates (fast, slow and control) were calculated based on the number of cleaved embryos from a specific group. Fast and slow cleavage and blastocyst rates were submitted to *t*-test.

The lipid composition as characterized by MALDI-MS for each developmental stage was submitted to multivariate and univariate statistical analyses. Only ions represented in ≥50% of the samples from each specific group or completely absent were used for further analysis. The intensity values of each peak, across multiple spectra, were normalized by the total ion count (TIC) technique. Partial least square discriminant analysis (PLS-DA) was performed using MetaboAnalyst 3.1 software to evaluate the relationship between variance in the data and differences among embryo development kinetics (FCL and SCL; FMO and SMO; FBL and SBL, FBL and VBL, SBL and VBL). For univariate analysis, *t*-tests were performed to indicate differences in relative abundance of the identified ions between specific groups from cleavage and 8–16-cell stages. Analysis of variance (ANOVA) and Tukey's post hoc test were applied to perform this evaluation for blastocysts and throughout the development. The alpha error was set to 5%.

Results

Embryo production

There was no difference ($P = 0.47$) in the cleavage rates (40 hpi) between the fast and slow embryo groups (FCL: 37.7 ± 1.7%; SCL: 33.3 ± 0.6%; total cleavage: 71.0 ± 1.7). Embryos from the fast group progressed more ($P = 0.028$) to the blastocyst stage than the slow ones (FBL: 24.6 ± 1.2%; SBL: 10.7 ± 1.9%; total blastocysts: 35.5 ± 2.5%).

MALDI-MS

Fast and slow embryos were analyzed at three time points (40, 112 and 186 hpi) by MALDI-MS to collect information about their lipid profiles. Representative MALDI-MS spectra for each group of samples are shown in Fig. 2. Here, PCs, SMs and triacylglycerol (TAG) ions were mainly observed. MALDI-MS data were explored by multivariate analysis to reveal differences in their relative abundances among groups. The results demonstrated a great separation between FMO and SMO, as well as for IVB, compared with *in vitro*-produced blastocysts with a slight samples overlap (Figs 4A and 5A). The embryos from FCL and SCL groups and the blastocysts derived from these presented similar profiles, not allowing a clear stratification (Fig. 3A). The ions that showed relevant differences among groups were attributed based on previous lipid profile studies of animal and human oocytes and embryos performed by MALDI-MS (Milne *et al.* 2006; Ferreira *et al.*, 2010; Sudano *et al.*, 2012, 2016; Jung *et al.*, 2014; Shrestha *et al.*, 2014). The assigned lipids are described in Supporting information Table S1 by class abbreviation followed by the total number of carbons and double bounds in the acyl residues attached to the glycerol backbone (in parentheses).

At 40 hpi, univariate analysis revealed a higher abundance of phospholipid ions – protonated SM (16:0), PC (32:0), PC (34:2),

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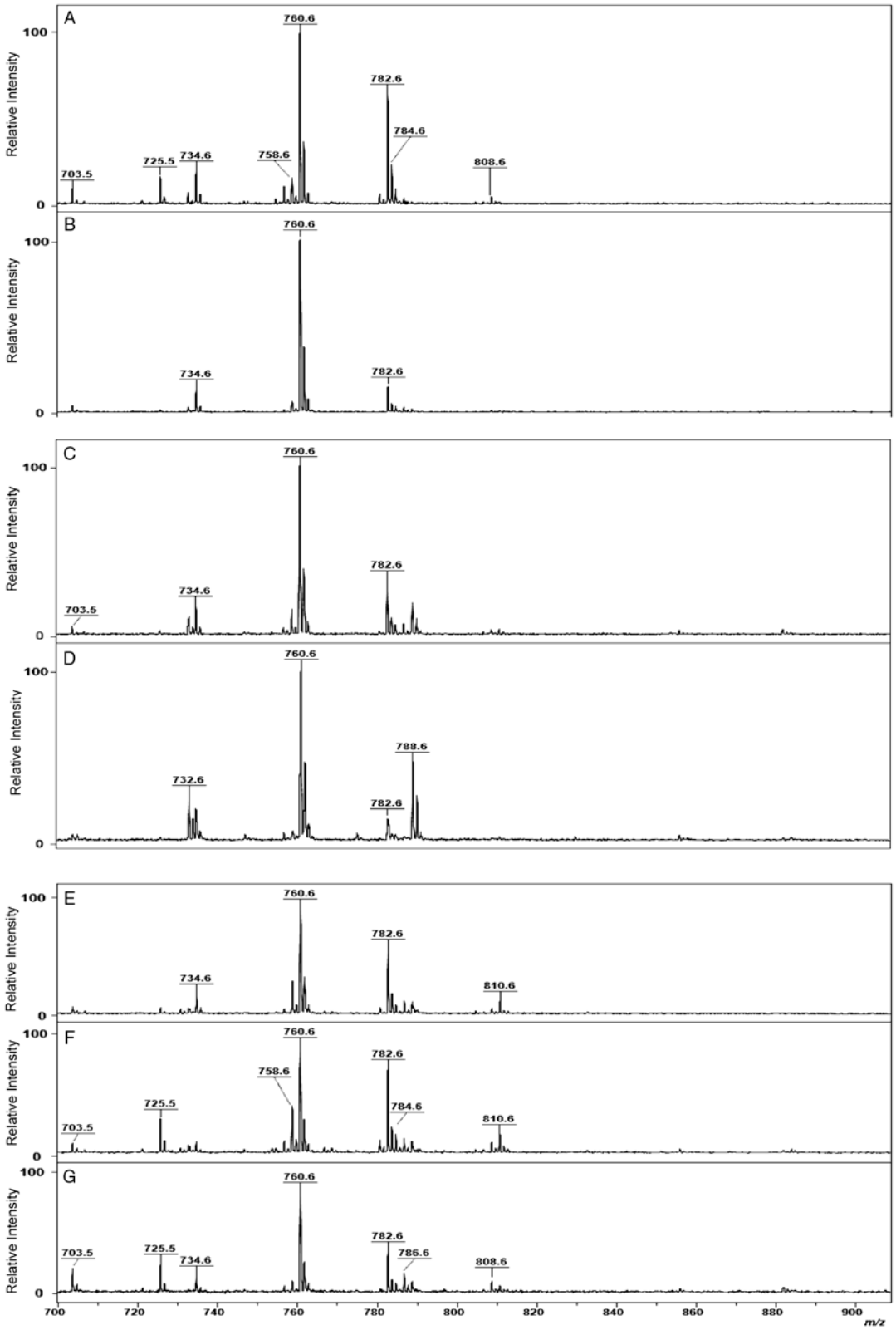

Figure 2. Representative matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) in positive ion mode of slow and fast cleaved *in vitro*-produced embryos at 40 hpi (slow cleavage (SCL) (*A*) and fast cleavage (FCL) (*B*), respectively); slow and fast *in vitro*-produced 8–16-cell embryos at 112 hpi (slow 8–16 cells (SMO) (*C*) and fast 8–16 cells (FMO) (D), respectively); slow and fast *in vitro*-produced blastocysts at 186 hpi (slow blastocyst (SBL) (*E*) and fast blastocyst (FBL) (*F*) respectively) and *in vivo*-derived blastocysts (VBL) (*G*).

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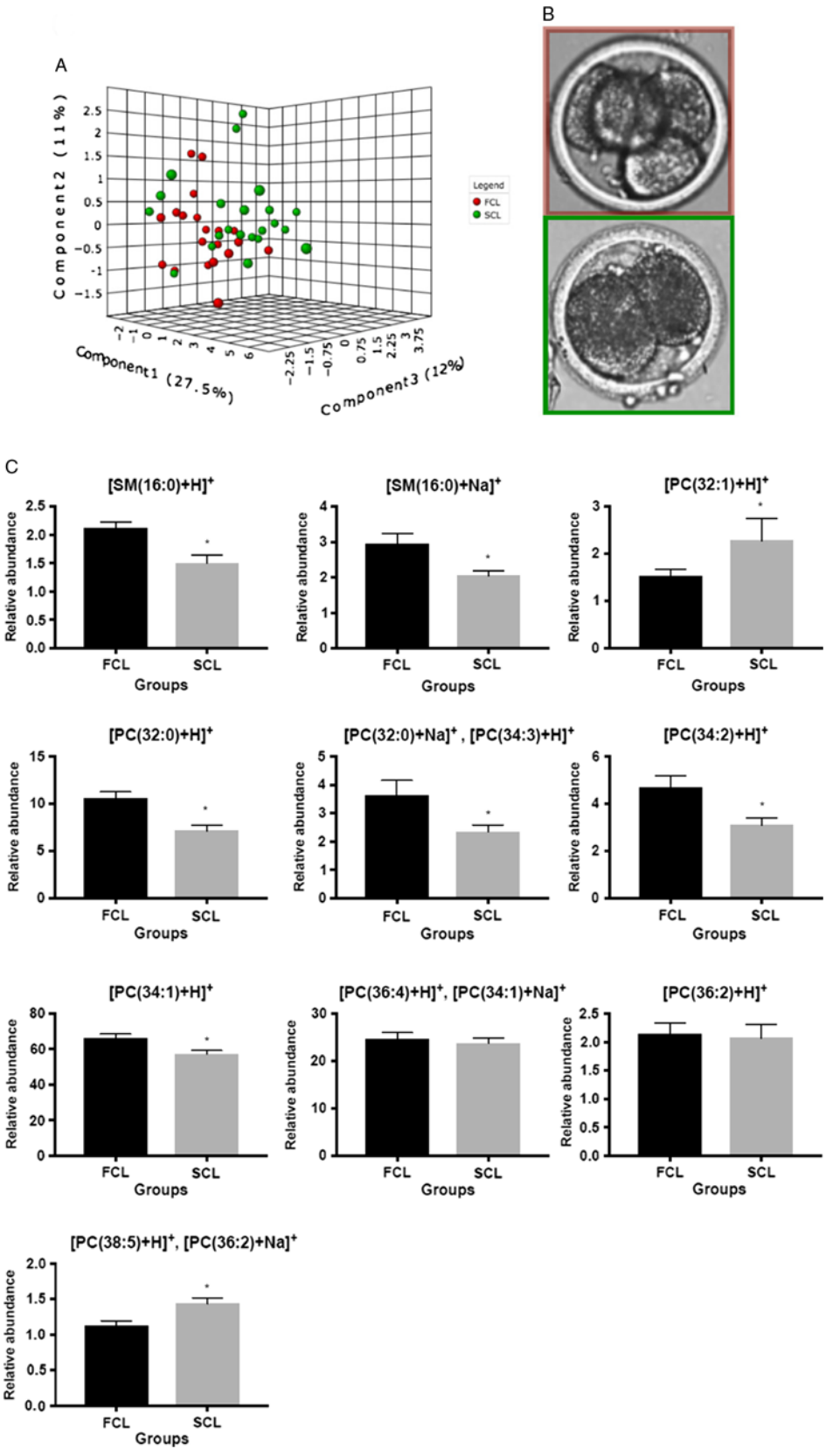

Figure 3. Data of embryos with different development kinetics (fast and slow) at 40 hpi. (*A*) Three-dimensional partial least square discriminant analysis (PLS-DA) plots for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). (*B*) Representative embryo morphology image. (*C*) Differentially expressed ions and their respective lipid classes. The number of fast cleavage (FCL) and slow cleavage (SCL) were 30 for each group. *Bars are significantly different at $P < 0.05$.

and PC (34:1); and sodiated SM (16:0) and PC (32:0) or protonated PC (34:3) – in fast embryos while the slow embryos presented a higher abundance of protonated PC (32:1) and protonated PC (38:5) or sodiated PC (36:2) (Fig. 3C).

At 112 hpi, the relative abundances of the PL – protonated PC (32:1), and PC (32:0), sodiated PC (32:0) or protonated PC (34:3), and protonated PC (38:4) or sodiated PC (36:1) – were reduced ($P < 0.05$) in the slow (SMO) compared with fast

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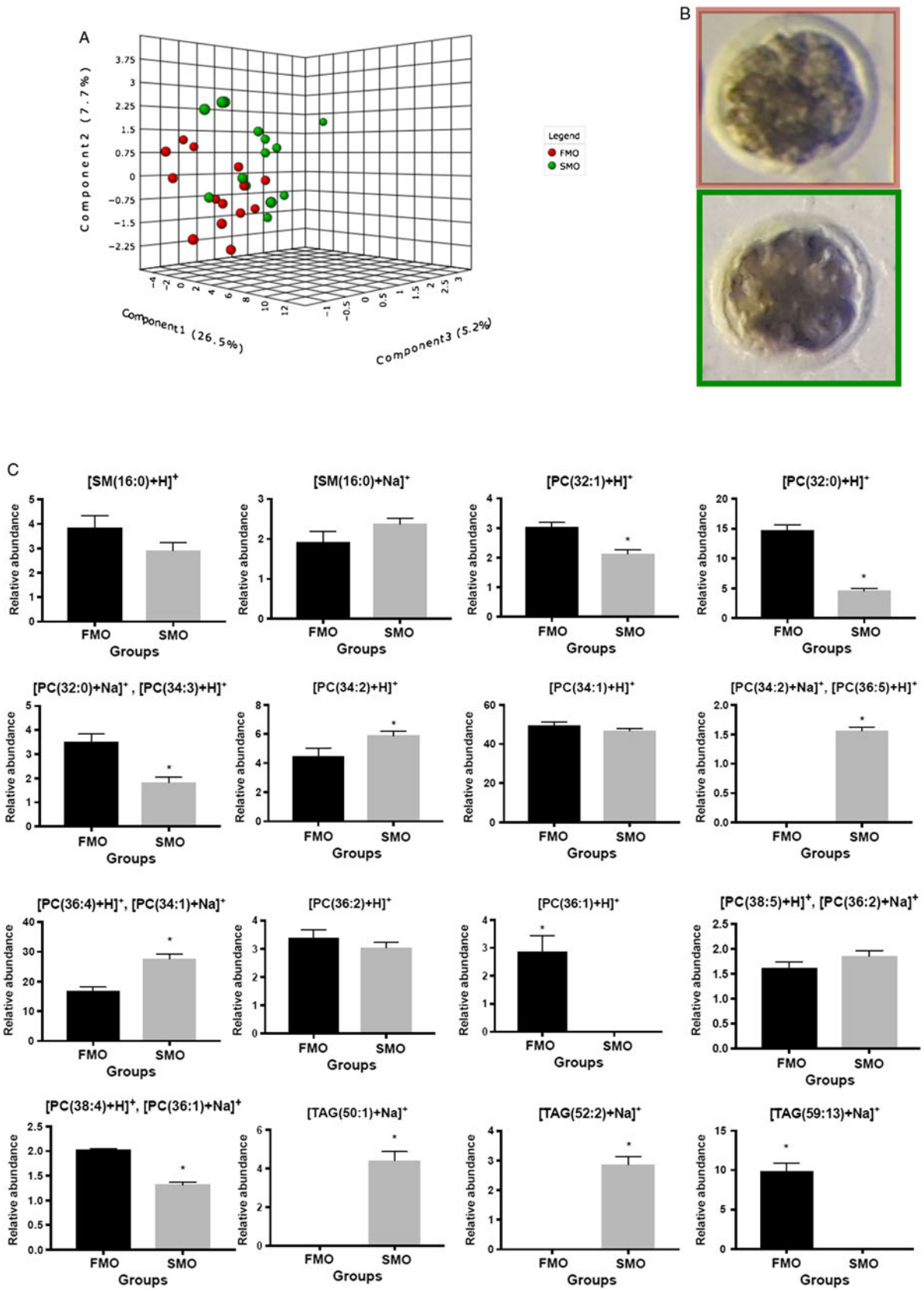

Figure 4. Data of embryos with different development kinetics (fast and slow) at 112 hpi. (*A*) Three-dimensional partial least square discriminant analysis (PLS-DA) plots for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). (*B*) Representative embryo morphology image. (*C*) Differentially expressed ions and their respective lipid classes. The number of fast 8–16 cells (FMO) and slow 8–16 cells (SMO) were 30 for each group. *Bars are significantly different at $P < 0.05$.

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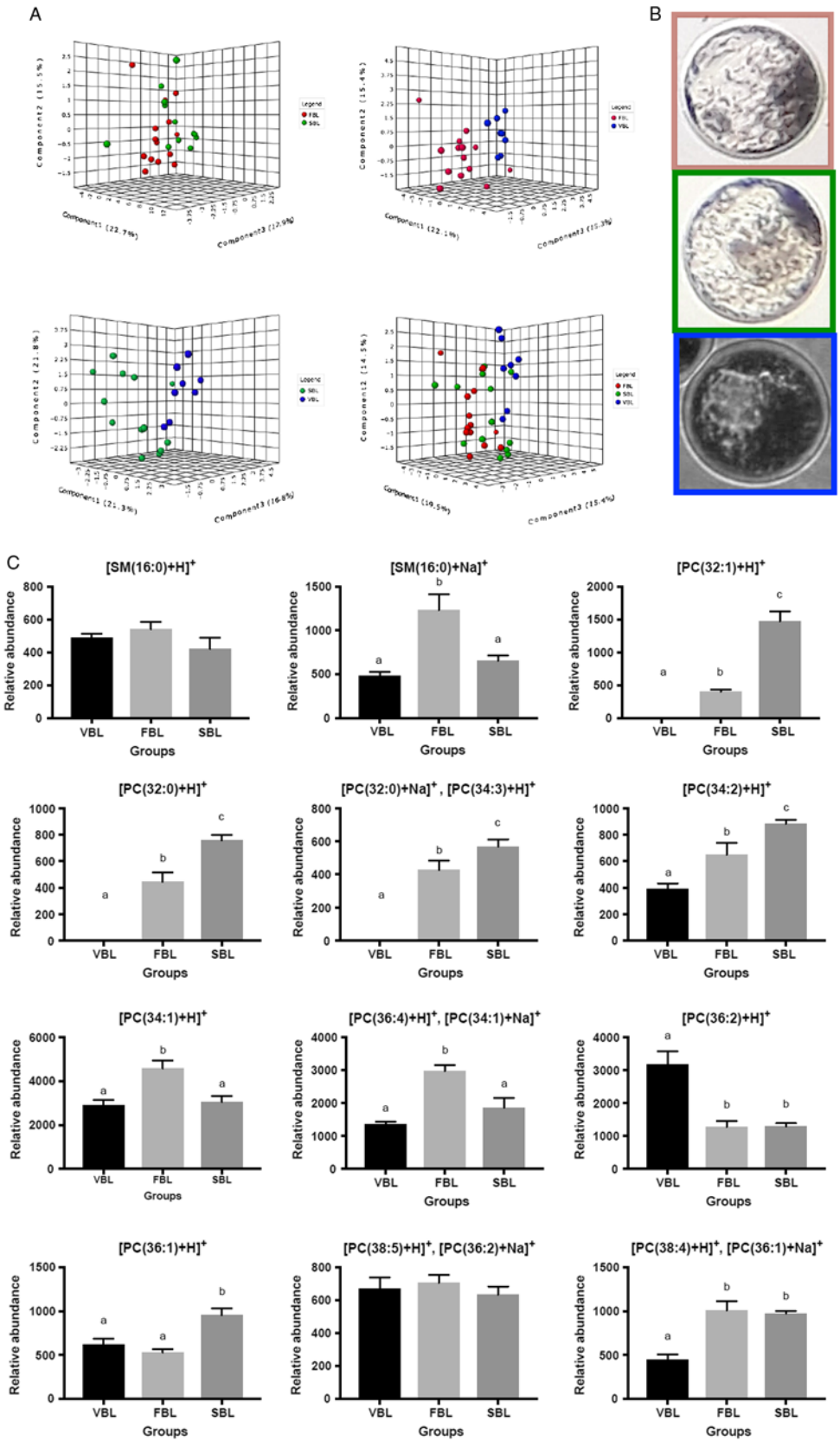

Figure 5. Data of embryos with different development kinetics (fast and slow) at 186 hpi and *in vivo* blastocysts. (*A*) Three-dimensional partial least square discriminant analysis (PLS-DA) plots for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). (*B*) Representative embryo morphology image. (*C*) Differentially expressed ions and their respective lipid classes. The numbers of fast blastocysts (FBL), slow blastocysts (SBL) and *in vivo*-derived blastocysts (VBL) were respectively 30, 30 and 10. [a,b,c]Bars with different superscript indicate significant differences at $P < 0.05$.

(FMO) group. The relative abundances of protonated PC (34:2) and protonated PC (36:4)or sodiated PC (34:1) were reduced ($P < 0.05$) in the fast (FMO) compared with the slow (SMO) group. One PL and TAG were specific for the FMO group [PC (36:1) + H]$^+$ and [TAG (59:13) + Na]$^+$, and one PL and two TAG were specific for the SMO group [PC (34:2) + Na]$^+$ or [PC (36:5) + H]$^+$, [TAG (50:1) + Na]$^+$ and [TAG (52:2) + Na]$^+$ (Fig. 4C).

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At the blastocyst stage (186 hpi), significant changes in the abundance of 10 ions (one SM and nine PC) were observed by univariate analysis. The sodiated SM (16:0), protonated PC (34:1), and protonated PC (36:4) or sodiated PC (34:1) were more represented in the FBL group than in the VBL and SBL groups. The protonated PC (32:1), PC (32:0), and PC (34:2) and sodiated PC (32:0) or protonated PC (34:3) were lower or absent in VBL, presenting higher levels in SBL. The protonated PC (36:2), PC (36:1) and protonated PC (38:4) or sodiated PC (36:1) were overrepresented in VBL, SBL and FBL/SBL groups, respectively (Fig. 5C).

Discussion

The kinetics of embryo development has been used to select human embryos with higher viability (Herrero and Meseguer, 2013). In bovine, embryos that cleave later have a lower capacity to form a blastocyst, although they show the same ability to establish pregnancy (Lonergan *et al.*, 1999). Corroborating with this expectation, we observed a decrease in blastocyst formation when the second cellular division was delayed. Despite the influence of morphokinetics on blastocyst formation, the kinetics of the early cleavages is also related to energy metabolism, oxygen consumption and epigenomic status (Leese, 2012; Market-Velker *et al.*, 2012; Milazzotto *et al.*, 2016; Ispada *et al.*, 2018).

Lipid content is directly related to embryonic competence and development (Leibo *et al.*, 1996; Abe *et al.*, 2002; Kuleshova and Lopata, 2002). In our study, the lipid profile was found to be dependent on embryonic stage and development kinetics and phospholipids (PL) were the major class of lipids identified.

During early cleavages (40 hpi), as for 112 and 186 hpi, PCs were the most affected class of ions by the kinetics of development. PCs are important ionic initiators of lipid metabolism such as phosphatidic acid (PA), arachidonic acid (AA) and diacylglycerols (DAG), which are essential signalling molecules during the first stages of embryonic development (Das and Rand, 1984; Goñi and Alonso, 1999). Changes in PCs can affect many plasma membrane properties such as fluidity, permeability, and thermal phase behaviour (Edidin, 2003), which are essential features for cell viability and cryopreservation success (Leibo *et al.*, 1996; Abe *et al.*, 2002; Kuleshova and Lopata, 2002).

At the 8–16-cell (112 hpi) stage, the differences in the lipid profiles between fast and slow embryos were more pronounced. This period is crucial for bovine embryos development, as it is the moment at which embryonic genome activation takes place (Sirard, 2012). During this phase, apoptosis can occur in embryos in response to a non-optimized environment, leading to embryo blocking (Vandaele *et al.*, 2007).

Some evidence of this response is related in a previous study, in which caspase activity was more pronounced in this period, mainly in slow embryos, and in which total caspase and caspases 3 and 7 were increased when compared with fast embryos. In addition, slow embryos showed a lower blastocyst rate and higher levels of ceramide in the culture medium, suggesting that these embryos were more susceptible to stress (Silva *et al.*, 2016). Cells undergoing apoptosis have an accelerated SM catabolism by sphingomyelinase and an increase in ceramide, which is related to the permeability of cells in the membrane lipid bilayers. In addition, ceramide acts as a 'coordinator' of the stress response in eukaryotes (reviewed by Jung *et al.*, 2014).

Despite that fact that SMs were similar in FMO and SMO, SMs (16:0) were higher in FCL, suggesting a better quality stress response in this group, favouring genome activation and development of embryos with this kinetic pattern. In fact, slower embryos had a higher caspase activity (2–4 cells at 40 hpi) than the fastest ones (5–8-cells at 40 hpi), suggesting higher cellular stress at the beginning of slow embryo development, with possible consequences during embryonic genome activation. Also, fast cleavage embryos had altered gene expression of the important transcripts and a higher blastocyst conversion when compared with slow cleavage embryos due to the embryonic arrest, impairing the development for later stages (Lonergan *et al.*, 1999; Fair *et al.*, 2004; Dode *et al.*, 2006; Vandaele *et al.*, 2007).

Two triacylglycerols (TAG) ions were also revealed at the 112 hpi stage that were specific for the SMO group. TAGs are the main constituents of lipid droplets in the cell cytoplasm (Ferguson and Leese, 1999). This class of lipids may have an important role as energy suppliers during early embryo development (Ferguson and Leese, 2006).

Regarding the blastocyst stage, PLS-DA analysis indicated that the SBL and FBL groups (*in vitro*) have similar membrane lipid profiles in comparison with the VBL group (Fig. 4C). Differences in the lipid profiles between IVP and *in vivo*-derived bovine embryos had already been described (González-Serrano *et al.*, 2013). In the present work, the profile of protonated PC (32:0), and sodiated PC (32:0) or protonated PC (34:3), protonated PC (34:1) and PC (36:1) were ions specific for the *in vitro* groups, reinforcing the idea that a non-optimized culture system influences lipid metabolism.

Despite the differences cited above, in this study embryos from the SBL group cultured in a medium supplemented with FBS, were subtly more close to *in vivo*-derived blastocysts. An example was the sodiated SM (16:0), which was more represented in FBL than VBL and SBL. SM molecules have a high affinity for cholesterol and these two lipids pack tightly into liquid-ordered domains forming lipid rafts, indicating that embryos from different groups may present differences regarding membrane characteristics (Van Meer *et al.*, 2008; Milhas *et al.*, 2010)

In conclusion, different embryo development kinetics were able to be resolved by their lipid profiles and a greater difference in membrane lipids was revealed at the 112 hpi stage, a critical moment during early embryonic development. Although the lipid profile of the slow blastocysts group is slightly closer to the *in vivo*-derived blastocysts, *in vitro*-produced blastocysts, independently of kinetics, still influenced by a non-optimized environment. Therefore, changes in the IVP system are necessary to support the development of more viable blastocysts.

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Conflict of interest. The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

Ethical standards. Not applicable.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199419000534.

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