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Phosphatidylcholine and Sphingomyelin Profiles Vary in *Bos taurus indicus* and *Bos taurus taurus* In Vitro- and In Vivo-Produced Blastocysts[1]

Mateus J. Sudano,[2,4] Vanessa G. Santos,[5] Alessandra Tata,[5] Christina R. Ferreira,[5] Daniela M. Paschoal,[4] Rui Machado,[6] José Buratini,[7] Marcos N. Eberlin,[5] and Fernanda D.C. Landim-Alvarenga[3,4]

[4]*Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), Botucatu, Brazil*
[5]*ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas, Campinas, Brazil*
[6]*Embrapa Southeast Cattle, São Carlos, Brazil*
[7]*Department of Physiology, São Paulo State University (UNESP), Botucatu, Brazil*

ABSTRACT

Lipid droplets, subspecies (*Bos taurus indicus* vs. *Bos taurus taurus*), and in vitro culture are known to influence cryopreservation of bovine embryos. Limited information is available regarding differences in membrane lipids in embryo, such as phosphatidylcholines (PC) and sphingomyelins (SM). The objective of the present study was to compare the profiles of several PC and SM species and relate this information to cytoplasmic lipid levels present in Nellore (*B. taurus indicus*) and Simmental (*B. taurus taurus*) blastocysts produced in vitro (IVP) or in vivo (ET). Simmental and IVP embryos had more cytoplasmic lipid content than Nellore and ET embryos (n = 30). Blastocysts were submitted to matrix-assisted laser desorption/ionization mass spectrometry. Differences in the PC profile were addressed by principal component analysis. The lipid species with PC (32:1) and PC (34:1) had higher ion abundances in Nellore embryos, whereas PC (34:2) was higher in Simmental embryos. IVP embryos had less abundant ions of PC (32:1), PC (34:2), and PC (36:5) compared to ET embryos. Moreover, ion abundance of PC (32:0) was higher in both Nellore and Simmental IVP embryos compared to ET embryos. Therefore, mass spectrometry profiles of PC and SM species significantly differ with regard to unsaturation level and carbon chain composition in bovine blastocysts due to subspecies and in vitro culture conditions. Because PC abundances of Nellore and Simmental embryos were distinct (34:1 vs. 34:2), as were those of IVP and ET embryos (32:0 vs. 36:5), they are potential markers of postcryopreservation embryonic survival.

cryopreservation, cryotolerance biomarkers, embryo, in vitro culture, lipidomics, MALDI-MS, mass spectrometry, taurine, zebuine

INTRODUCTION

Embryo cryopreservation is an assisted reproductive technology that allows storage of excess embryos derived from in vitro production and embryo transfer programs so they can be commercialized or transferred at the most convenient time. Commercial in vitro production of embryos is performed on a large scale for Nellore and other zebuine (*Bos taurus indicus*) breeds due to their high oocyte yield with ultrasound-guided follicular aspiration [1]. Embryos produced in vitro (IVP) are, however, more sensitive to cryopreservation than those produced in vivo (ET); despite many advances in embryo research and commercial IVP embryos over past decades, cryopreservation remains one of the most challenging areas of bovine embryo biotechnology [2, 3].

Cytoplasmic lipid droplet levels are associated with reduced embryo survival after cryopreservation. Bovine IVP embryos have more cytoplasmic lipid droplets than ET embryos and as a result have reduced postcryopreservation survival [3–5]. The fatty acid profile obtained from extraction of the total pool of embryo lipids can be correlated with breed, in vitro culture conditions, and cryopreservation [6–8]. Lipid research has therefore become a major focus of research in this field [3, 4, 7, 9].

How lipid accumulation occurs in IVP embryo cell cytoplasm is unknown. Cytoplasmic lipid accumulation can occur as a result of fetal calf serum (FCS) supplementation in culture media [4, 9, 10] or abnormalities in energy metabolism. Accumulation can also be influenced with drugs that act on lipidogenic pathways [5, 11–13].

Triglycerides (TAG) are the major lipid present in the cytoplasm of mammalian cells as lipid droplets [6, 7, 14]. These lipids are a stored energy supply for oocytes and embryos [15]. However, phospholipids (PL) are the most abundant lipid in eukaryotic cell membranes, and their role in successful cryopreservation remains poorly understood [16]. PL, most particularly phosphatidylcholines (PC) and sphingomyelins (SM), are structural units of functional membranes, and their composition determines most physicochemical cell membrane properties, including fluidity, permeability, and thermal phase behavior [17].

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) allows chemical structural analysis of PC and SM species in individual bovine embryos [18]. This technique has revealed that protein supplementation and atmospheric conditions of embryo during in vitro culture alter profiles of these lipid species [18].

The objective of the present work was therefore to associate the amount of cytoplasmic lipid droplets present in Nellore and

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[2]Correspondence: Mateus José Sudano, São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science (FMVZ), Department of Animal Reproduction and Veterinary Radiology, Rubião Jr. s/n°, Botucatu, SP 18618–970, Brazil. E-mail: mjsudano@gmail.com
[3]Correspondence: Fernanda da Cruz Landim-Alvarenga, São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science (FMVZ), Department of Animal Reproduction and Veterinary Radiology, Rubião Jr. s/n°, Botucatu, SP 18618–970, Brazil. E-mail: fernanda@fmvz.unesp.br

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Simmental IVP or ET blastocysts with their PC and SM profiles, which were obtained using MALDI-MS. We observed significant differences in a number of lipid species due to subspecies (*B. taurus indicus* and *Bos taurus taurus*) and origin (IVP and ET), suggesting that some PC species might be used as biomarkers of the higher postcryopreservation survival observed in Simmental and ET embryos.

MATERIALS AND METHODS

Experimental Design

A 2 × 2 factorial experiment design was used to test the effect of two subspecies—*B. taurus indicus* (Nellore) and *B. taurus taurus* (Simmental)—and two origins—IVP and ET—on lipid content and MS fingerprint profiles. Single bovine embryos were used as the experimental unit in 8–30 replications.

Reagents Used

All materials were acquired from Sigma-Aldrich Corp., except when otherwise indicated.

Weather, Animals, and Feed

The local weather is tropical (CwA Köppen classification), characterized by a rainy, hot summer and a dry winter. Nonlactating mature Nellore (*B. taurus indicus*; n = 21; age, 4–10 yr; body wt, 446 ± 18 kg; values are least squares mean ± SD) and Simmental (*B. taurus taurus*; n = 22; age, 3–6 yr; body wt, 498 ± 21 kg; values are least squares mean ± SD) cows were used. All animals were considered to be sound after gynecological examination. Cows were managed under an intensive rotational grazing system based on highly productive, artificially fertilized tropical pastures. All animals had free access to mineral supplement and water.

In Vitro Production of Nellore and Simmental Blastocysts

Ultrasound-guided follicular aspiration. Ovum pick-up procedures were carried out on visible follicles 2 mm or larger in diameter using a B-mode ultrasound scanner (Mindray Bio-Medical Electronics Co., Ltd.), equipped with a 7.5-MHz convex array transducer fitted into the intravaginal device (WTA Watanabe Tecnologia Aplicada), and a stainless-steel guide. Follicular puncture was performed using a disposable 19-gauge, 3 1/2-inch hypodermic needle (BD) connected to a 50-ml conical tube (Corning) via a silicon tube. Aspiration was performed using a vacuum pump (Watanabe, Ltd.) with a negative pressure of 10–12 ml water/min. The collection medium was PBS (Nutricell) supplemented with 1 ml/L of heparin (Hemofol; Cristalia).

In vitro maturation. The aspirated content was immediately filtered, and oocytes were washed three times in TCM 199 with Earle salts (Gibco, Invitrogen Co.) supplemented with 25 mM Hepes, 10% FCS, 100 μg/ml of streptomycin sulfate, and 100 IU/ml of penicillin (Gibco). Only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were used for the experiments. Selected oocytes were matured in vitro by incubation at 38.5°C in 5% CO_2 in air with saturated humidity for 24 h. Drops containing 90 μl of TCM 199 with Earle salts and L-glutamine (Gibco) supplemented with 5 mg/ml of bovine serum albumin (BSA; fatty acid free; A-8806; Sigma), 0.2 mM sodium pyruvate, 5 mg/ml of luteinizing hormone (LH; Lutropin-V; Bioniche), 1 mg/ml of follicle-stimulating hormone (FSH; Folltropin-V; Bioniche), 100 μg/ml of streptomycin sulfate, and 100 IU/ml of penicillin (Gibco), containing 20–30 oocytes each, were placed in Petri dishes and covered with mineral oil.

In vitro fertilization. At the end of the maturation period, groups of 20–30 oocytes were transferred to drops containing 90 μl of fertilization media, covered with mineral oil. Nellore and Simmental oocytes were subjected to in vitro fertilization (IVF) with frozen semen from a sample of each bull breed with proven fertility. Sperm were selected by the Percoll method [19], and the concentration was adjusted to 2×10^6 sperm/ml. Fertilization occurred in human tubal fluid (Irvine Scientific) supplemented with 5 mg/ml of BSA, 0.2 mM pyruvate, 30 μg/ml of heparin, 18 μM penicillamine, 10 μM hypotaurine, 1.8 μM epinephrine, 100 μg/ml of streptomycin sulfate, and 100 IU/ml of penicillin (Gibco). Oocytes and sperm were incubated under the same conditions as with in vitro maturation for approximately 18 h. The day of fertilization was defined as Day 0.

In vitro culture. Eighteen hours post-IVF, presumptive zygotes were denuded by repeated pipetting and transferred to culture plates in drops containing 90 μl of culture media (n = 20–30 structures/drop), covered with mineral oil, and cultured in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The culture medium used was synthetic oviduct fluid medium (SOFaa) [20] with 2.7 mM *myo*-inositol, 0.2 mM pyruvate, low FCS supplementation (2.5%; to avoid deleterious effect as described previously [3], because FCS is a protein supplement added in most culture media used for bovine embryo research and commercial production), 5 mg/ml of BSA, 100 μg/ml of streptomycin sulfate, and 100 IU/ml of penicillin. After 60 h of culture, cleavage was checked, and structures that were not cleaved were discarded. Embryos were transferred to new 90-μl drops of the aforementioned culture media. Embryos remained in this condition until Day 7, when blastocyst production was evaluated. Samples were stained with Sudan black B, or MALDI-MS profiles were acquired.

In Vivo Production of Nellore and Simmental Blastocyts

Embryos were collected from adult, nonlactating Nellore (*B. taurus indicus*, n = 7) and Simmental (*B. taurus taurus*, n = 8) cows with good body condition scores. The ovarian superstimulation protocol was based on that described by Barros and Nogueira [21]. On Day 0 (without regard to stage of the estrous cycle), cows received an intravaginal insert containing 1.9 g of progesterone (CIDR; Pfizer Animal Health) and 2 mg of estradiol benzoate i.m. (Estrogin; Farmavet). Starting on Day 4, cows received 200 mg of FSH from porcine pituitary (Folltropin-V) i.m. twice a day in eight decreasing doses (40%, 30%, 20%, and 10%). On Day 6, two doses of 150 μg of sodium cloprostenol (Ciosin; Intervet/Schering-Plough Animal Health) were given i.m. 12 h apart. The CIDR was removed 36 h after the first application of cloprostenol, and 12.5 mg of LH (Lutropin-V) were given i.m. 48 h after the first dose of cloprostenol. Nellore and Simmental cows were artificially inseminated (12 and 24 h after LH) with frozen semen of proven fertility from the same Nellore and Simmental bull used for IVF in the in vitro experimental groups. Embryo recovery (n = 45 and 39, respectively, for Nellore and Simmental cows) was carried out through uterine flushing with a PBS solution 7.5 days after the first artificial insemination, as previously described [22].

Semiquantitative Lipid Assay

A sample of blastocysts (n = 15 per group) was randomly selected during experimental replications and stained with Sudan black B, a lipophilic dye, as previously described [3]. These embryos were fixed in a 10% formaldehyde solution for 2 h at room temperature, washed, and transferred to drops of 50% ethanol. After 2 min, embryos were stained in drops of 1% Sudan black B (w/v) in 70% ethanol for 1–2 min and washed in 50% ethanol followed by 0.05% polyvinyl alcohol in distilled water. Prepared embryos were mounted in 10 μl of glycerol on cover slips and examined under a light microscope at 600× magnification. To estimate the relative amount of lipid content, a photograph was taken of each embryo and submitted to Image J 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Embryo color pictures were converted to a gray-scale image (Fig. 1). Embryos were delimited to obtain area (μm^2) and gray intensity mean (arbitrary units), and gray intensity per area was calculated (arbitrary units/μm^2). Because comparison of embryo lipid content must be performed in terms of volume (μm^3), the amount of gray intensity per area was converted to gray intensity per volume (arbitrary units/μm^3) using the formula gray intensity/volume = (gray intensity/area)$^{1.5}$.

Lipid Analysis by MALDI-MS

Sample preparation. Each Nellore and Simmental IVP and ET embryo was washed five times in drops of PBS solution and stored in microtubes containing 2–4 μl of PBS at −80°C until analysis, when samples were thawed by pipetting 100 μl of a 1:1 (v/v) solution of methanol (ACS/HPLC grade; Burdick and Jackson)/ultrapure water (Millipore) into the microtube and washed five times in the same solution. Each embryo was placed on a unique spot on the MALDI target plate under the stereomicroscope. Samples were allowed to dry at room temperature, and their locations were recorded. Just before analysis, 1 μl of 1.0 M 2,5-dihydroxybenzoic acid diluted in pure methanol was deposited on each target spot to cover the embryos, and the spots were allowed to dry at room temperature.

MALDI-MS data acquisition. MALDI-MS and MALDI-MS/MS data were acquired in the positive-ion and reflectron modes using an Autoflex III MALDI time-of-flight mass spectrometer (Brucker Daltonics) equipped with smart-beam laser technology. The MS data were acquired in the 700-1200 *m/z* range by averaging 1500 consecutive laser shots with a frequency of 200 until signals in the region of interest were observed and then disappeared due to the consumption of the sample. MALDI-MS/MS data were manually acquired by increasing the collision energy until extensive dissociation of the precursor ion was observed. Argon was used as the collision gas. FlexAnalysis 3.0 software (Brucker Daltonics) was used to check the mass spectra. The most intense ions that were clearly distinct from noise after the exclusion of isotopic peaks were

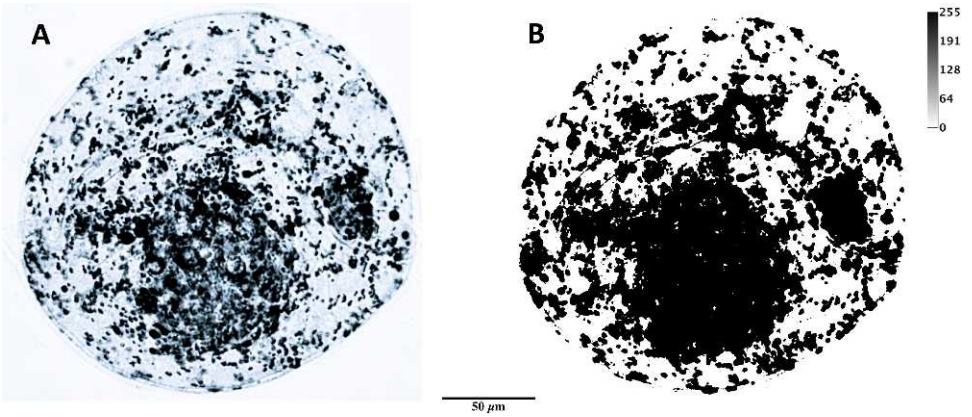


FIG. 1. Optical view of an embryo stained with Sudan black B (**A**) and gray-scale conversion for semiquantitative lipid content assay (**B**). Original magnification ×600.

considered from each spectrum and used as the starting point to search for *m/z* values corresponding to lipids.

Lipid assignment. MALDI-MS/MS laser-induced fragmentation technique (LIFT) analysis was performed to confirm the structure of lipid species that were significant for experimental group differentiation. To increase the intensity of the signal and simplify the isolation of the parent ions, a pool of 10 embryos was placed on each spot. The LIFT data, previously obtained by our lab [18], and two lipid databases (http://lipidsearch.jp and http://www.lipidmaps.org) were utilized to assign PL species (PC and SM).

Statistical Analysis

For lipid content analysis, data were analyzed with ANOVA using the generalized linear mixed model (GLIMMIX) procedure with the SAS statistical software package (Version 9.2; SAS Institute, Inc.). Sources of variation in the model included subspecies (*B. taurus indicus* [Nellore] vs. *B. taurus taurus* [Simmental]), origin (IVP vs. ET), and first-order interactions; all factors were considered to be fixed effects. If the ANOVA was significant, means were separated using the Tukey test. The data are reported as the least-squares means ± SEM. In the absence of significant interactions, only main effect means are presented.

For lipid MS profiles, multivariate and univariate statistical models were used. A first principal component analysis (PCA) was performed using Pirouette v.3.11 (Infometrix, Inc.) and the MetaboAnalyst website [23]. Based on the loading results from PCA, the most important ions that explained the higher variance of the data were selected and submitted to ANOVA using the GLIMMIX procedure with SAS software to better study the significance of each ion for all groups. Sources of variation in the model included subspecies (*B. taurus indicus* [Nellore] vs. *B. taurus taurus* [Simmental]), origin (IVP vs. ET), and first-order interactions; all factors were considered to be fixed effects. In the absence of significant interactions, only main effect means are presented.

RESULTS

Lipid Content by Sudan Black B

Simmental embryos had higher ($P < 0.05$) lipid content than Nellore embryos (Table 1 and Fig. 2). ET embryos had less ($P < 0.05$) cytoplasmic lipid than IVP embryos (Table 1 and Fig. 2). The majority of lipid droplets in ET blastocysts occurred in the inner cell mass (ICM) as compared to trophoblast cells, but a random distribution of lipid droplets was found in both the ICM and trophoblast cells of the IVP blastocysts (Fig. 2).

PC and SM Species Detected by MALDI-MS

Phospholipids are labeled by class abbreviation (PC or SM), followed by the total number of carbons and double bonds attached to the glycerol backbone, separated by a colon (see Table 2). PL structures were previously attributed based on MALDI-MS lipid profile studies as described by Ferreira et al. [18] as well as two lipid databases (http://lipidsearch.jp and http://www.lipidmaps.org). Figure 3 displays a representative lipid profile of each group. More than one isomer can occur for a single lipid.

Principal component analysis shows that the four experimental groups can be resolved via their MALDI-MS profiles, because the two-dimensional PCA plots cluster samples corresponding to different breeds (Nellore vs. Simmental) and origins (IVP vs. ET), with little overlap between Nellore and Simmental IVP groups (Fig. 4A). In the three-dimensional PCA plot, however, Nellore and Simmental IVP and ET embryos were clearly separated, with even more pronounced group individualization (Fig. 4B). The most significant lipids indicated by the PCA are in Table 2.

Main Effect of Subspecies on the Lipid Profile of Embryos

Subspecies mainly affected three lipid ions, *m/z* 732.5, 758.6, and 760.6, corresponding to protonated PC (32:1), PC (34:2), and PC (34:1), respectively. PC (32:1) and PC (34:1)

TABLE 1. Main effect means of subspecies and origin on cytoplasmic lipid content expressed by gray intensity, gray intensity per area, and gray intensity per volume (least-squares mean ± SEM, n = 30 per group).

	Subspecies			Origin		
Parameter	*Bos taurus indicus* (Nellore)	*Bos taurus taurus* (Simmental)	*P*-value	IVP	ET	*P*-value
Gray intensity	141.6 ± 5.3[a]	161.7 ± 5.9[b]	0.017	169.4 ± 5.6[A]	133.9 ± 5.6[B]	<0.001
Gray intensity per area ($\times 10^{-4}/\mu m^2$)	38.1 ± 2.8[a]	50.8 ± 3.1[b]	0.005	53.5 ± 3.0[A]	35.3 ± 2.9[B]	<0.001
Gray intensity per volume ($\times 10^{-4}/\mu m^3$)	2.4 ± 0.3[a]	3.4 ± 0.3[b]	0.008	4.0 ± 0.3[A]	2.1 ± 0.3[B]	<0.001

[a,b] Within a row, subspecies means without a common superscript differ significantly ($P < 0.05$).
[A,B] Within a row, origin means without a common superscript differ significantly ($P < 0.05$).

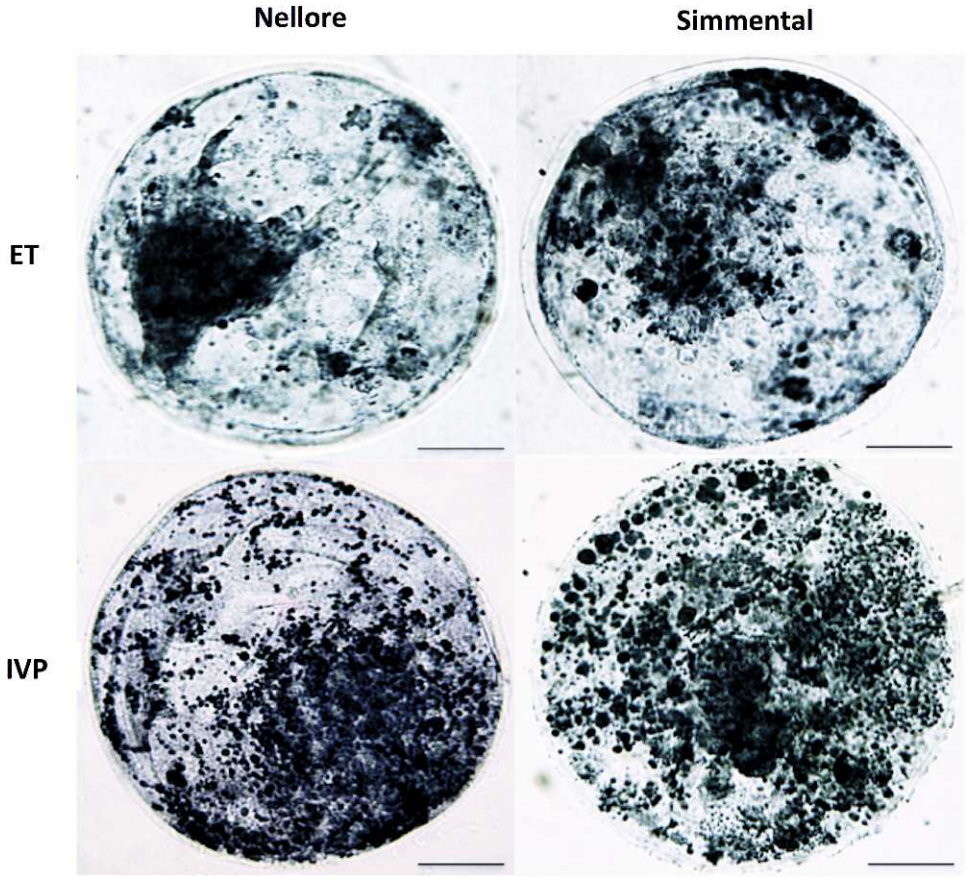


FIG. 2. Light micrographs of Nellore and Simmental ET and IVP embryos stained with Sudan black B. Black areas indicate sudanophilic cytoplasmic lipid droplets. Original magnification ×600; bar = 50 μm.

presented with higher abundance ($P < 0.05$) in Nellore embryos, whereas the PC (34:2) abundance was higher ($P < 0.05$) in Simmental embryos (Fig. 5A). The abundance of ion *m/z* 802.6 was not different ($P > 0.05$) between Nellore and Simmental embryos.

Main Effect of Origin in the Lipid Profile of Embryos

Origin also affected three lipid species, detected via the ions *m/z* 732.5, 758.6, and 802.6, corresponding to protonated PC (32:1), PC (34:2), and sodiated PC (36:5), respectively. The abundance of these three lipid ions was elevated ($P < 0.05$) in ET embryos compared with the IVP group (Fig. 5B).

Interaction Effect Between Subspecies and Origin in the Lipid Profile of Embryos

Interactions between the subspecies and origin affected the lipid profile of the four experimental groups in different ways. Lipid ions of *m/z* 703.5, 734.6, and 786.6 (Fig. 6), which were attributed to protonated SM (16:0), PC (32:0), and PC (36:2), respectively, were different when comparing IVP and ET embryos in both Simmental and Nellore breeds. SM (16:0) and PC (36:2) ion abundances were increased ($P < 0.05$) in Simmental ET embryos, whereas PC (32:0) was significantly higher in the IVP embryos of both breeds.

The lipid ions of *m/z* 782.6 [PC (34:6) + $H]^+$ and/or [PC (34:1) + $Na]^+$ and 810.6 [PC (38:4) + $H]^+$ and/or [PC (36:1) + $Na]^+$ were less abundant ($P < 0.05$) in Nellore ET embryos compared with the other three groups (Fig. 6). Similarly, abundance of SM (16:0) and PC (34:0) ions was higher ($P < 0.05$) in Simmental ET embryos compared with IVP embryos. Nellore ET embryos also had a low abundance of SM (16:0) compared with Simmental IVP embryos (Fig. 6).

Nellore ET embryos had the highest abundance ($P < 0.05$) of protonated PC (36:1) (*m/z* 788.6). This lipid ion level was also significantly higher in Nellore IVP embryos than in Simmental IVP embryos (Fig. 6).

MS/MS Characterization of PL in the Embryos

To substantiate lipid attributions based on literature and database search, lipid ions were subjected to MS/MS via the LIFT-MS technique [24]. Fragmentation patterns of lipid ions are known to provide class structural attribution confirmation [18, 25–27], and those observed were compatible with both PC and SM. The loss of a neutral (NL) of 59 Da is due to neutral trimethylamine [$N(CH_3)_3$], whereas loss of 124 Da is related to the cyclophosphane ring ($C_2H_5O_4P$). The fragment ion *m/z* 147 corresponds to sodiated cyclophosphane and that of *m/z* 184 to monoprotonated dihydrogen phosphate choline ($C_5H_{15}PO_4N$) (Fig. 7).

DISCUSSION

In the present study, we have associated the cytoplasmic lipid content of bovine blastocysts with the chemical structure of their membrane lipids, discriminating between *B. taurus indicus* and *B. taurus taurus* (represented by Nellore and Simmental breeds, respectively) and also between IVP and ET embryos.

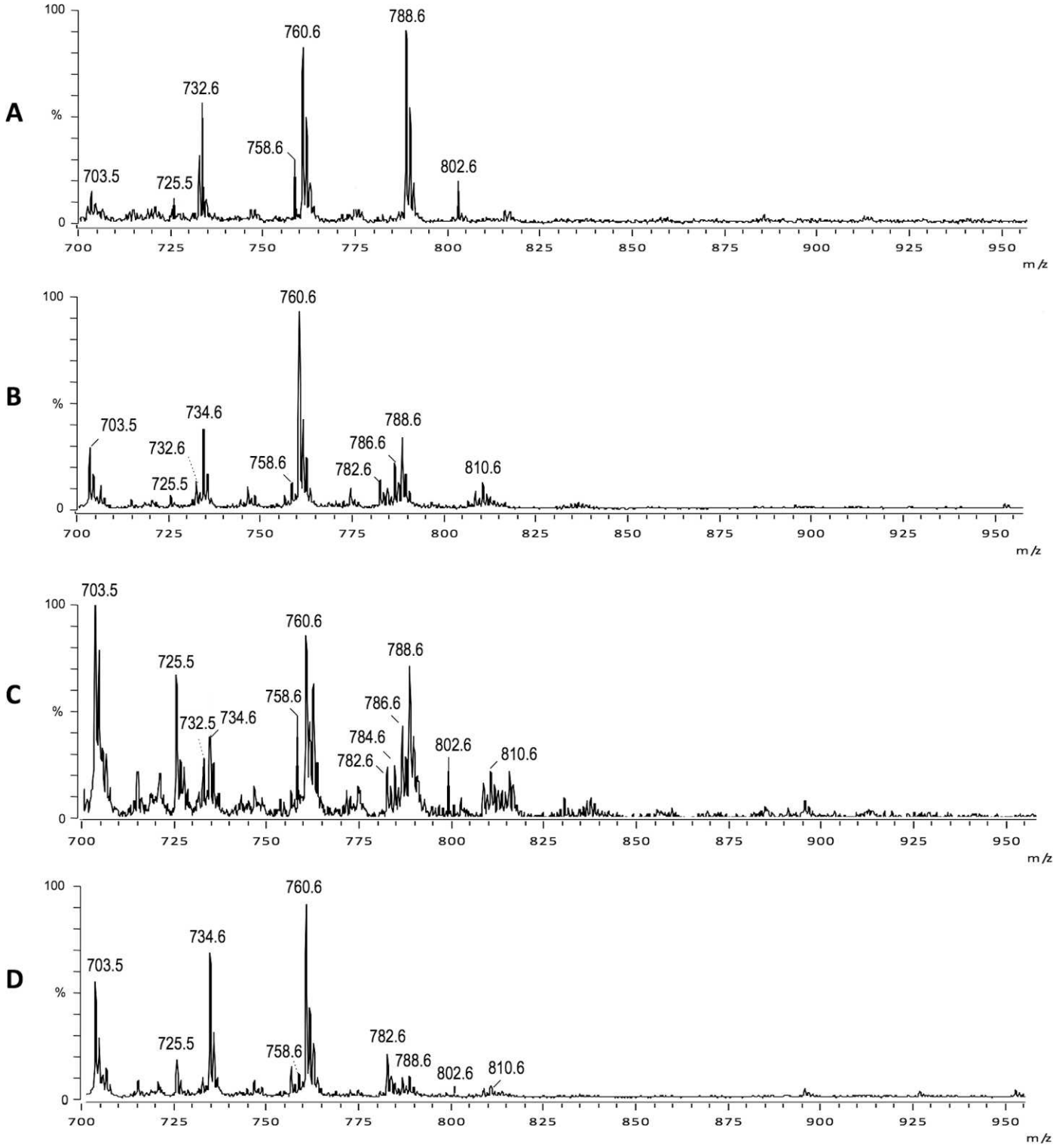


FIG. 3. Representative MALDI-MS in the positive-ion mode for Nellore and Simmental ET and IVP individual embryos. **A**) Nellore ET embryo. **B**) Nellore IVP embryo. **C**) Simmental ET embryo. **D**) Simmental IVP embryo.

Lipids play a significant role in energy storage, cell structure, modification of physical properties, and function of biological membranes; they also have potent effects on cell-cell interactions, cell proliferation, and intra- and intercellular transport [28, 29]. A series of studies on lipid composition in bovine embryos has examined fatty acid TAG and lipid content composition [4–6, 11, 30]. Lower postcryopreservation embryo survival has also frequently been associated with high embryo lipid content [4, 9, 10].

It remains unclear why cytoplasmic lipid accumulation affects embryonic cryotolerance; controversy persists in the literature. In an ultrastructural descriptive study, ET taurine (*B. taurus taurus*) embryos presented greater cytoplasmic lipid accumulation than zebuine (*B. taurus indicus*) embryos, but the

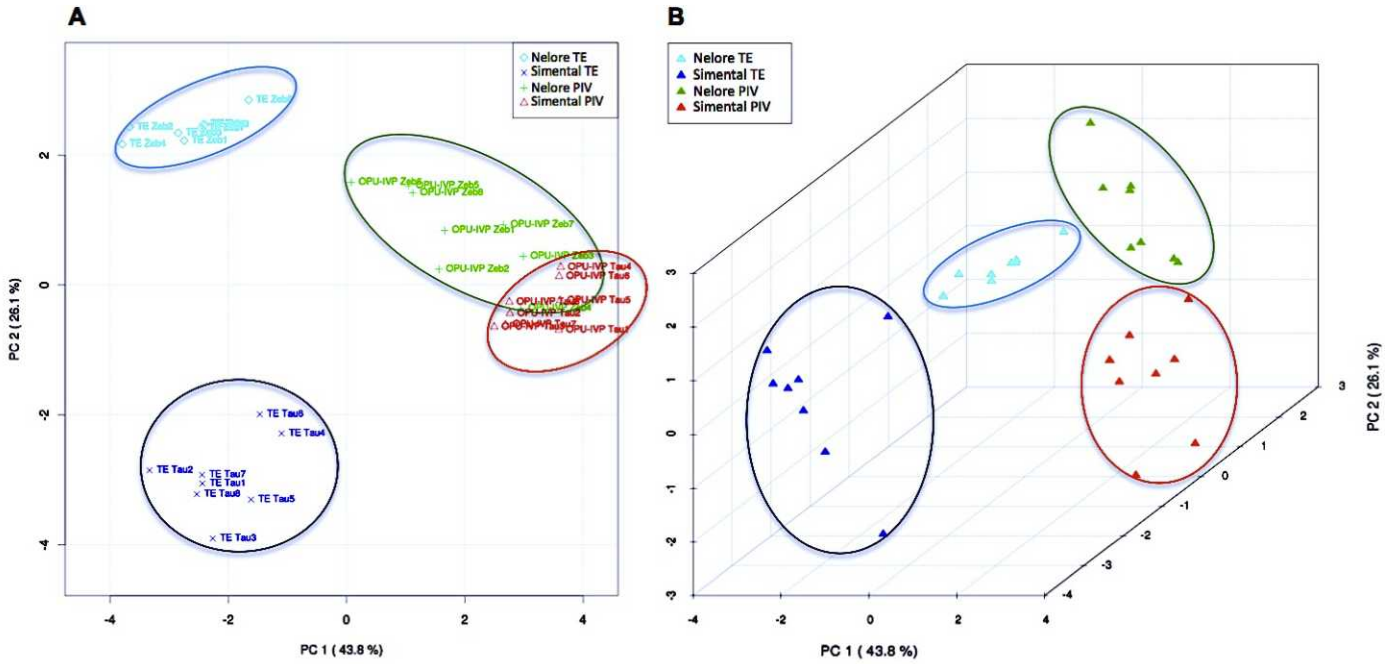


FIG. 4. Two-dimensional (**A**) and three-dimensional (**B**) PCA plots for MALDI-MS data of Nellore and Simmental ET and IVP embryos (n = 8 per group).

TABLE 2. Significant PC and SM indicated in PCA via MALDI-MS of individual Nellore and Simmental IVP and ET embryos.*

m/z	Lipid ion (carbons:unsaturation)
703.5	$[SM\ (16:0) + H]^+$
725.5	$[SM\ (16:0) + Na]^+$
732.5	$[PC\ (32:1) + H]^+$
734.6	$[PC\ (32:0) + H]^+$
758.6	$[PC\ (34:2) + H]^+$
760.6	$[PC\ (34:1) + H]^+$
782.6	$[PC\ (34:6) + H]^+$, $[PC\ (34:1) + Na]^+$
784.6	$[PC\ (34:0) + Na]^+$
786.6	$[PC\ (36:2) + H]^+$
788.6	$[PC\ (36:1) + H]^+$
802.6	$[PC\ (36:5) + Na]^+$
810.6	$[PC\ (38:4) + H]^+$, $[PC\ (36:1) + Na]^+$

* Identification based on LIFT data, our earlier results [18], and two lipid databases (http://lipidsearch.jp and http://www.lipidmaps.org).

former showed higher cryotolerance [31]. Arreseigor et al. [32] reported that taurine frozen ET embryos resulted in a higher pregnancy rate than with frozen ET zebuine embryos. In a previous study by our group, embryo quality represented by apoptosis better predicted embryo survival after vitrification than did lipid content [3, 33]. These results suggest that study of the actual influence of embryo lipid content on cryotolerance should be expanded to study membrane lipid composition, which is crucial for successful cryopreservation.

Preliminary data showed that Simmental embryos had higher postvitrification survival compared with Nellore embryos (34.6% vs. 20.2 %, respectively, of hatching/hatched; $P = 0.04$), and ET embryos had higher survival rate after vitrification compared with IVP embryos (38.5% vs. 18.1%, respectively, of hatching/hatched; $P = 0.004$) [34]. These data can be correlated with our Sudan black B staining data, which corroborate previous work [3, 5, 11, 31] indicating that *B. taurus taurus* embryos have higher lipid content than *B. taurus indicus* and that IVP methods, which involve in vitro culture, leads to more cytoplasmic lipid than with ET methods. In summary, the origin effect (IVP vs. ET) on lipid content can be correlated with a reduction in cryotolerance, but the subspecies effect contradicts the hypothesis that cytoplasmic lipid accumulation is prejudicial to cryopreservation.

Somatic cells take up fatty acids that are esterified and stored as lipid droplets [14]. A major biological function of these lipids is to serve as a storage form of metabolic energy [6,

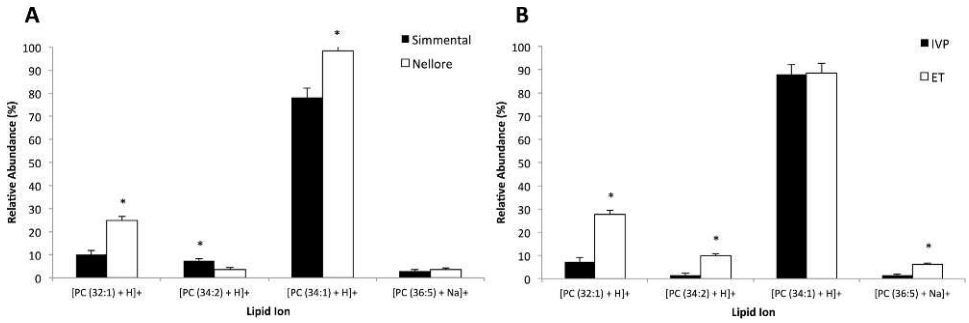


FIG. 5. Subspecies (**A**) and origin (**B**) effects on the relative intensity of lipid ions presented in the Nellore and Simmental IVP and ET embryos (least-squares mean ± SEM). Asterisk indicates differences between pairs of dark and light bars ($P < 0.05$, n = 16 per group).

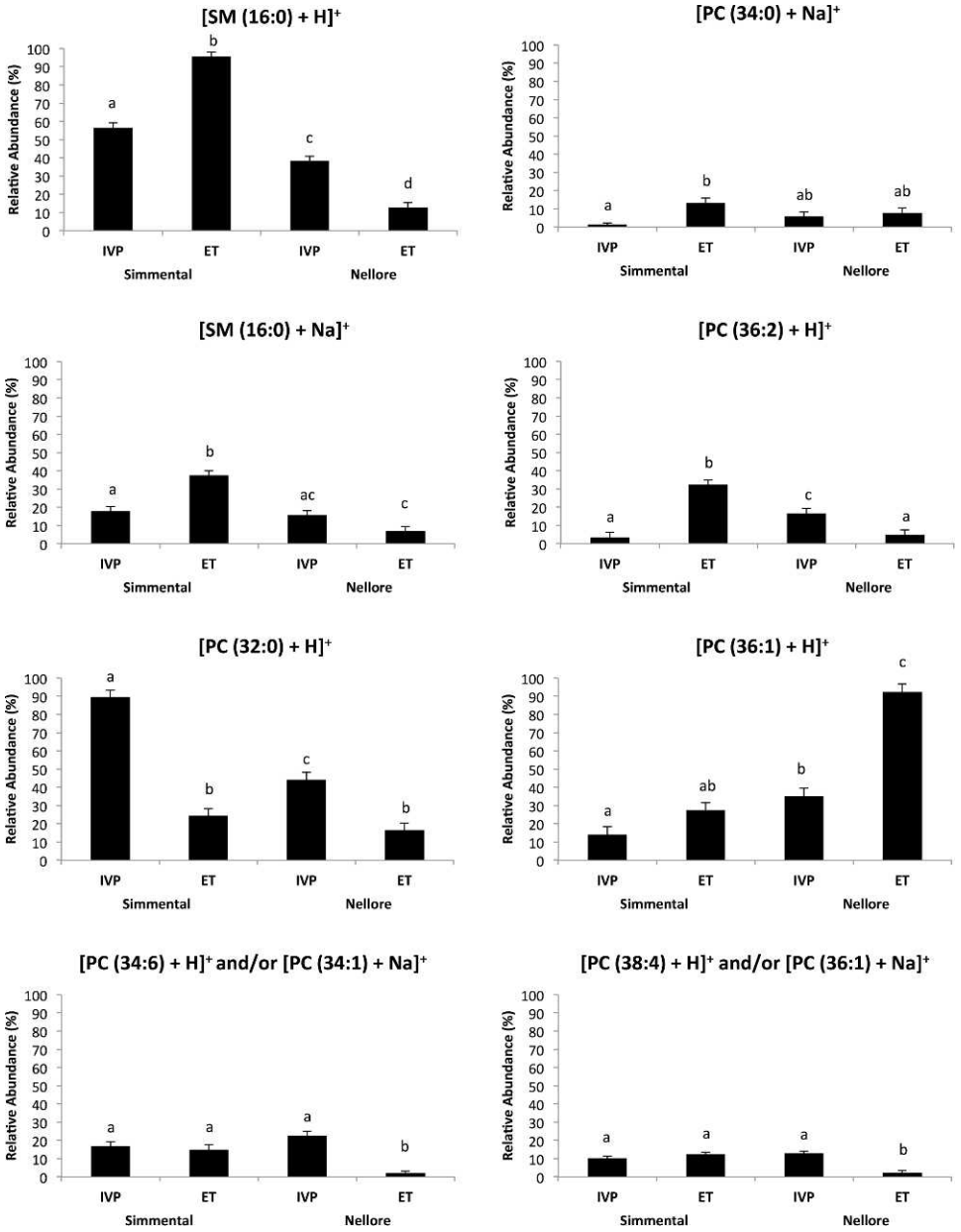


FIG. 6. Relative abundance of lipid ions present in Nellore and Simmental IVP and ET embryos. Values without common lowercase letters differ significantly ($P < 0.05$, n = 8 per group).

35, 36]. In the present study, the majority of lipid droplets in ET blastocysts occurred in the ICM, not the trophoblast cells (Fig. 2). The lipid droplet level decreases during embryo development [37–39], because lipids may be released from droplets during blastocyst formation. Lipid droplets are now envisaged as metabolically active organelles that provide an important endogenous energy source. The decrease in overall fat content during blastocyst formation may be explained by their utilization, mainly by trophoblastic cells, via β-oxidation [40] during blastocyst formation, expansion of the blastocoele, and hatching [35]. In IVP embryos, the increase in lipid droplets observed, and the even distribution of this organelle in trophoblasts and ICM, may indicate an imbalance in mitochondrial metabolism, with less utilization of the fat reservoir. However, the higher lipid content of Simmental embryo cytoplasm is difficult to explain.

It has already been reported that the cytoplasmic membrane is extremely sensitive to low temperature [41, 42] and that lipid composition influences its physical properties [29], particularly membrane fluidity. Understanding the membrane lipid profile is fundamental to addressing the difficulties of postcryopreservation embryo survival. Changes in the lipid structural composition of the membranes, which are difficult to detect using staining procedures, are readily detected by MS. These changes are associated with the impact of in vitro culture conditions, and especially with FCS supplementation in the culture media, and they result in negative effects on embryo survival after cryopreservation, as previously reported [3, 4, 6, 10, 33].

In lipidomics, MALDI-MS has proven to be a powerful tool [43], generating PL (mainly PC and SM in the positive-ion mode) and TAG profiles with simple interpretation. Previously, our group described a practical and novel approach for direct

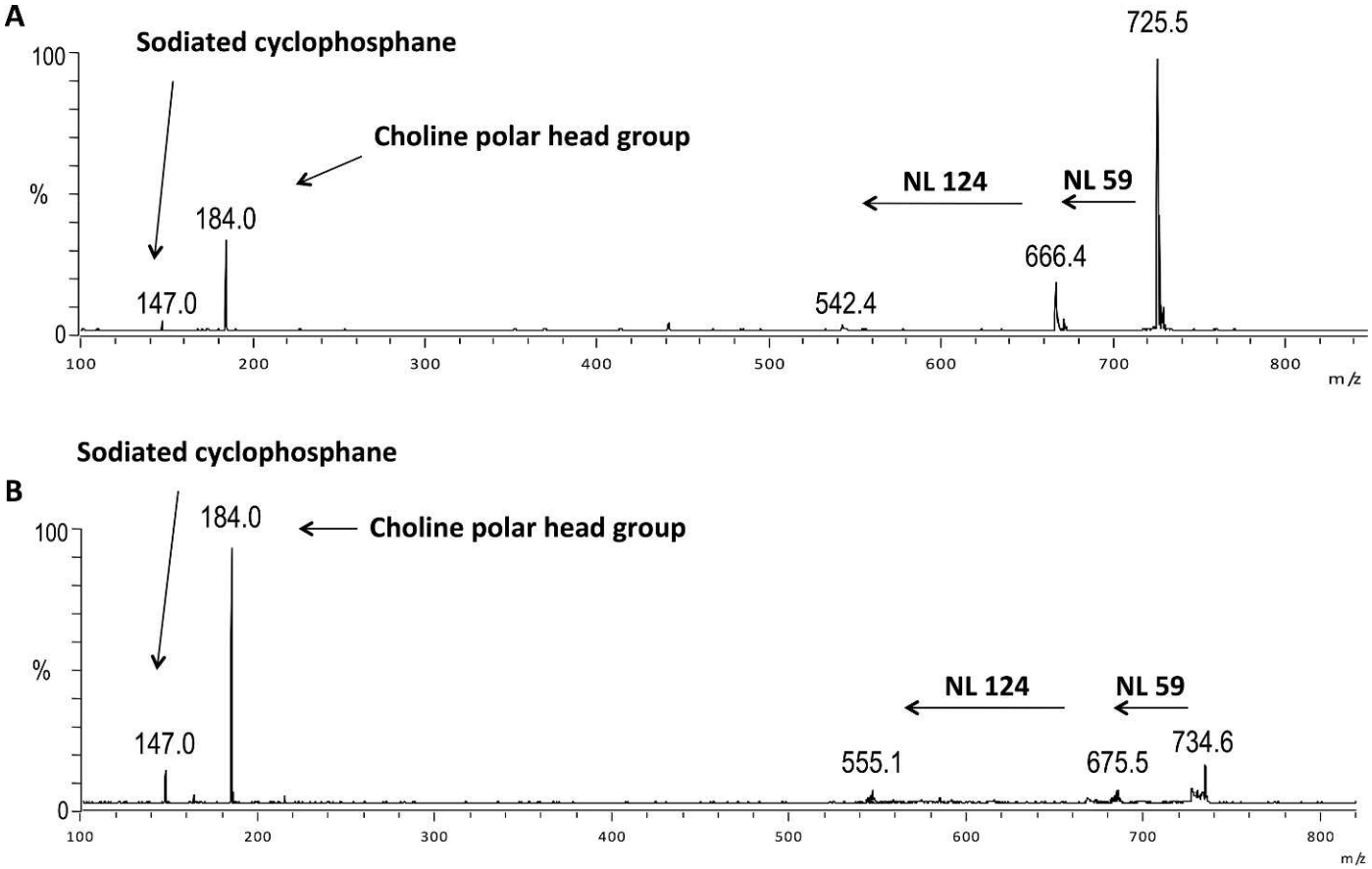


FIG. 7. MALDI-MS/MS for *m/z* 725.5 $[SM (16:0) + Na]^+$ (**A**) and *m/z* 734.6 $[PC (32:0) + H]^+$ (**B**). Both spectra display characteristic neutral loss (NL) of 59 Da (trimethylamine) and of 124 Da (cyclophosphane ring). Sodiated cyclophosphane (*m/z* 147.0) and choline polar head group (*m/z* 184.0) ions are also diagnostic.

analysis of single, intact embryos from various species using MALDI-MS in a procedure involving no extraction, chemical manipulation, or preparation. We have also detected PC profile changes in blastocysts cultured under different protein supplementation (BSA vs. FCS) and atmospheric pressure (5% vs. 20%) conditions [18].

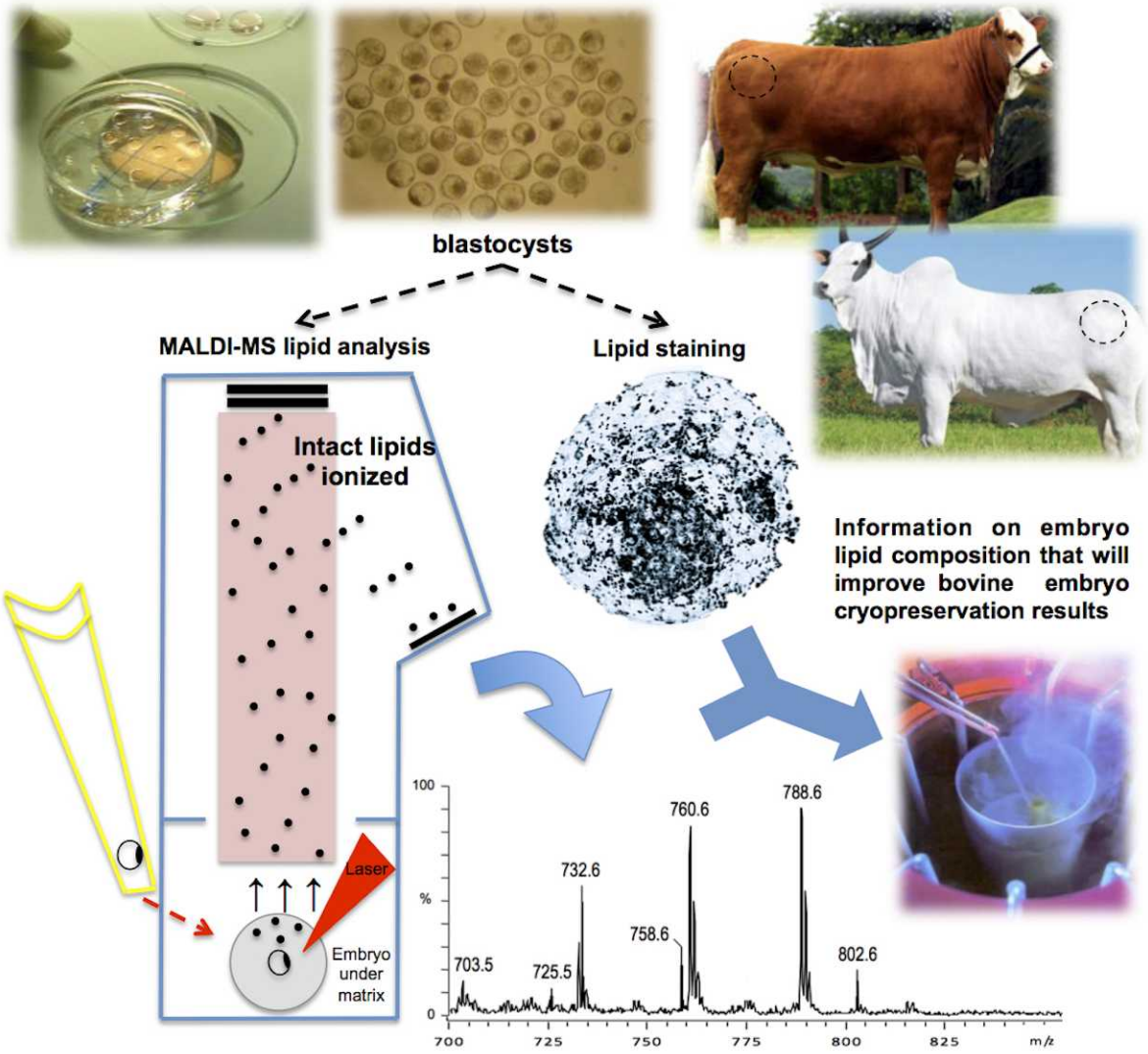
In the present study, MALDI-MS fingerprinting data of membrane lipids were analyzed by PCA, an established method [18, 44, 45], and representative spectra of each experimental group were identified. As expected, some overlap was found between Simmental and Nellore IVP embryos (Fig. 4A), possibly due to the impact of in vitro culture on lipid metabolism, which alters cell membrane properties and stability [46]. Confirming the hypothesis of membrane lipid differences, the number of carbons and fatty acid saturation are actively changing (Fig. 3). Despite the SM species having a low relevance for embryo postcryopreservation survival in the present study, this PL plays an important role in the heat shock-induced apoptosis through the SM pathway associated with the generation of ceramide [47]. However, higher membrane fluidity was expected with higher unsaturated lipid content, favoring postcryopreservation survival, and was observed in the Simmental and ET embryos (Figs. 5 and 8). Several studies have shown the benefits of unsaturated fatty acids for oocyte and embryo developmental competence and, especially, for postcryopreservation survival [7, 14, 48–52].

Simmental embryos favor PC containing linoleic (18:2) acid [PC (34:2)], whereas Nellore embryos favor PC containing palmitic (16:0) and stearic (18:0) acids [PC (32:1) and PC (34:1), respectively]. ET embryos favor PC containing oleic (18:1), linoleic (18:2) and linolenic (18:3) acids [PC (32:1), PC (34:2), and PC (36:5), respectively], whereas IVP embryos favor PC containing palmitic (16:0) and stearic (18:0) acids [PC (32:0) and PC (34:1), respectively].

Membrane fluidity is determined by PL composition, length of fatty acyl residues, number and position of the double bonds within PL, ratio of saturated to unsaturated fatty acid total content, cholesterol level, and protein content [28, 53–55]. The change or imbalance of one or more of these factors could lead to different rates of cryopreservation success. In the present study, we observed variations of unsaturation degree of PL fatty acyl residues and also differences in PL class composition. These results indicate how specific lipid species can affect the ability of embryos to survive after cryopreservation (Fig. 8).

In agreement with the influence of lipid composition on the cryoresponse, an increase in the degree of membrane lipid unsaturation during cold acclimation in plants [56], bacteria [57], fish [58], and mammals [28] has already been described. Additional double bonds in the membrane lipids increase membrane fluidity in lower ambient temperatures. The fact that *B. taurus taurus* have been naturally selected in Europe under cold temperatures might have determined the higher degree of unsaturated PL present in Simmental blastocysts.

In conclusion, a semiquantitative analysis of cytoplasmic lipids using Sudan black B staining showed that Simmental embryos had a higher cytoplasmic lipid content than Nellore embryos and that IVP embryos had more cytoplasmic lipids



Overview of MALDI-based selection of prospective lipid markers for cryopreservation survival based in the subspecies and origin (A), and in the interaction between subspecies and origin (B).

A	*m/z*	Lipid Ion (carbons:unsaturation)	Subspecies			Origin		
			Bos taurus (Simmental)	*Bos indicus* (Nellore)	Biomarker**	IVP	ET	Biomarker**
	732.5	PC (32:1)	↓	↑	negative	↓	↑	positive
	758.6	PC (34:2)	↑	↓	positive	↓	↑	positive
	760.6	PC (34:1)	↓	↑	negative	0*	0*	-
	802.6	PC (36:5)	0*	0*	-	↓	↑	positive
B	***m/z***	**Lipid Ion (carbons:unsaturation)**	**Simmental**			**Nellore**		
			IVP	**ET**	**Biomarker***	**IVP**	**ET**	**Biomarker***
	734.6	PC (32:0)	↑	↓	negative	↑	↓	negative

Arrows indicated elevated (↑) or reduced (↓) relative lipid ion abundance in relation to the other group.
* Indicated no differences of the relative lipid ion abundance between groups.
** Cryotolerance biomarkers were established based on the post-cryopreservation embryo survival.
- Undetermined.

FIG. 8. MALDI-MS analysis of lipids complements lipid staining data. By comparing Simmental and Nellore IVP and ET embryos, we observed consistent changes in membrane lipids that can be used as biomarkers to predict cryopreservation outcomes.

than ET embryos. The PCA of MALDI-MS lipid profiles allowed clustering of the four experimental groups according to the membrane lipids (PC and SM species). In summary, the most significant lipids—PC (32:0), PC (34:1), PC (34:2), and PC (36:5)—seem to function as prospective biomarkers for cryopreservation success with regard to subspecies, origin, and interactions between these factors (Fig. 8). The present data suggest that the assessment of lipid chemical composition may be a valuable tool for the development of in vitro culture systems that allow higher postcryopreservation survival of bovine blastocysts.

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