UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

CRIOTOLERÂNCIA DE EMBRIÕES *BOS TAURUS INDICUS* E *BOS TAURUS TAURUS* PRODUZIDOS *IN VITRO* E *IN VIVO*

MATEUS JOSÉ SUDANO

Botucatu, São Paulo
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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para a obtenção do título de Doutor

Orientador: Prof[a] Dr[a] Fernanda da Cruz Landim
Co-Orientador: Prof. Dr. José Buratini Junior

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"The whole of science is nothing more than a refinement of everyday thinking"

Essays in Physics, Albert Einstein
Philosophical Library, 1950.

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RESUMO

O objetivo deste experimento foi estudar os mecanismos envolvidos na sobrevivência à criopreservação de embriões *Bos taurus indicus* (Nelore) e *Bos taurus taurus* (Simental) produzidos *in vitro* (PIV) e *in vivo* (TE). Em um arranjo fatorial 2 x 2, duas subespécies (*Bos taurus indicus* vs *Bos taurus taurus*) e duas origens (PIV vs TE) foram utilizadas para testar a hipótese de que a capacidade de sobrevivência após a criopreservação é um evento multifatorial, e que a composição lipídica e o padrão de expressão gênica global afetam a criotolerância embrionária. Vacas Nelore (N=14) e Simental (N=14) foram submetidas a OPU e os oócitos recuperados (N=840 e 450, respectivamente) foram submetidos a MIV, FIV e CIV. Além disso, vacas Nelore (N=7) e Simental (N=8) foram submetidas a superestimulação ovariana, IATF e lavagem uterina. Os embriões PIV (N=349 e N=105) e TE (N=80 e N=74), respectivamente Nelore e Simental, foram submetidos as técnicas de vitrificação (N=70-94), coloração de Sudan Black (n=30), espectrometria de massas por ionização e dessorção a laser assistida por matriz (MALDI-MS; N=8 por grupo), microarray genômico (Nelore PIV N=4, Nelore TE N=4, Simental PIV N=3, Simental TE N=3) e validação no qPCR (N=8 por grupo). Modelos univariados (PROC LOGISTIC e PROC GLIMMIX- SAS 9.2) e multivariados (análise dos componentes principais-PCA- Piruete v.3.11 e MetaboAnalyst) foram utilizados na análise estatística. A análise do Microarray genômico procedeu-se no FlexArray 1.6.1.1. Genes com “fold change” de 1,5 e P≤0,05 foram considerados diferentemente expressos. Embriões Simental apresentaram maior sobrevivência após a vitrificação que os Nelore (34,6 vs 20,2%; P<0,05). Apesar disso, os embriões Simental apresentaram maior conteúdo lipídico do que os Nelore (3,4±0,3 vs 2,4±0,3 intensidade de cinza x 10^{-4} / μm^3 ; P<0,05). Quando se compararam embriões TE em relação aos PIV observou-se que os primeiros apresentaram melhor resposta à vitrificação (38,5 vs 18,1%; P<0,05)

e menor conteúdo lipídico (2,1±0,3 vs 4,0±0,3 intensidade de cinza x 10^{-4} / μm^3; P<0,05), em relação aos embriões PIV. Além disso, o perfil de lipídeos de membrana do tipo fosfocolinas (PC) e esfingomielinas (SM) foi característico para cada grupo; sugerindo marcadores positivo [PC (34:2) e PC (36:5)] e negativo [PC (32:0) e PC (34:1)] da criotolerância embrionária, respectivamente. Na análise do microarray genômico um total de 158, 532 e 53 genes foram diferencialmente expressos (P<0,05) entre as subespécies, origens e interação subespécies*origens, respectivamente. Genes envolvidos no metabolismo lipídico (*AUH* e *ELOVL6*), anti-apoptose (*DAD1*), e mitocondrial (*ATP5B*) estavam "upregulated" nos embriões Simental. O gene *GPX4*, envolvido no estresse oxidativo, estava "upregulated" nos embriões Nelore. Genes envolvidos no metabolismo lipídico (*ACSL3* e *ACSL6*), pró-apoptose (*DAP*), reconhecimento materno da gestação (*INTF2*), estresse térmico (*HSPA5*) e diferenciação celular e formação da placenta (*KRT18*) estavam "upregulated" nos embriões PIV. Além disso, houve interação significativa entre subespécies*origens nos genes relacionados à prenhez (*PAG2*) e apoptose (*PRDX2*). Conclui-se que diversas vias metabólicas fundamentais de sinalização celular e embrionária envolvidas em diferentes processos biológicos afetam a sobrevivência dos embriões após criopreservação.

Palavras- chave: criopreservação, composição lipídica, metabolismo lipídico, lipidômica, MALDI-MS, espectrometria de massas, transcriptômica, microarray.

SUDANO, M. J. **Cryotolerance of *Bos taurus indicus* and *Bos taurus taurus* in vitro and in vivo produced embryos.** Botucatu, 2013. 117p. Dissertação (Mestrado) – School of Veterinary Medicine and Animal Science, Botucatu city, São Paulo State University.

ABSTRACT

The objective was to study mechanisms involved in the post-cryopreservation survival of *Bos taurus indicus* (Nellore) and *Bos taurus taurus* (Simmental) in vitro (IVP) and in vivo (ET) produced embryos. In a 2x2 factorial experimental design, two subspecies (*Bos taurus indicus* vs *Bos taurus taurus*) and two origins (PIV vs ET) were used to test the hypotheses that the post-cryopreservation embryo survival capacity is a multifactorial event and that the lipid composition and the global gene expression pattern affect embryo cryotolerance. Nellore and Simmental cows (N=14) were submitted to OPU sessions and recovered oocytes (840 and 450, respectively) underwent IVM, IVF and IVC. Moreover, Nellore (N=7) and Simmental (N=8) cows were submitted to ovary superstimulation, FTAI, and uterine flushing. The IVP (N=349 and N=105) and ET (N=80 and N=74) embryos, Nellore and Simmental respectively, were used in the vitrification (N=70-94), Sudan Black B stain (N=30), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (N=8 per group), genomic microarray (Nellore IVP N=4, Nellore ET N=4, Simmental IVP N=3, Simmental ET N=3) and qPCR (N=8 per group) techniques. Univariate (PROC LOGISTIC and PROC GLIMMIX, SAS 9.2) and multivariate (PCA - Piruete v.3.11 and MetaboAnalyst) models were used for the statistical analysis. Microarray data analysis was performed in FlexArray 1.6.1.1 software. Genes with a fold change of at least 1.5 and a probability of $P<0.05$ were considered differentially expressed. Simmental embryos had higher post-vitrification survival (34.6 vs 20.2%; $P<0.05$) compared with Nellore. Nevertheless, Simmental embryos had higher lipid content than Nellore (3.4±0.3 vs 2.4±0.3 gray intensity x 10^{-4} / μm^3; $P<0.05$). When compared ET with PIV embryos, it was observed that the former had higher post-vitrification survival (38.5 vs 18.1%; $P<0.05$) and lesser lipid content (2.1±0.3 vs 4.0±0.3 gray intensity x 10^{-4} / μm^3; $P<0.05$). Additionally, membrane lipids profile (phosphocholine- PC and sphingomielins- SM) were characteristic of each group, and potential positive [PC (34:2) and PC (36:5)] or negative [PC (32:0)

and PC (34:1)] lipid biomarkers of embryo cryotolerance were established. Through microarray analysis, a total of 158, 532 and 53 genes were differentially (P<0.05) expressed between subspecies, origins and subspecies*origins interaction, respectively. Genes involved in lipid (*AUH* and *ELOVL6*), anti-apoptotic events (*DAD1*), and mitochondrial (*ATP5B*) metabolism were upregulated in taurine embryos. *GPX4* gene, involved in the oxidative stress, was upregulated in zebuine embryos. Genes involved in lipid metabolism (*ACSL3* and *ACSL6*), pro-apoptotic events (*DAP*), maternal recognition of pregnancy (*ITFN2*), heat shock (*HSPA5*) and cell differentiation and placenta formation (*KRT18*) were upregulated in IVP embryos. In addition, there was a significant subspecies*origins interaction in the genes related to pregnancy (*PAG2*) and apoptosis (*PRDX2*). In conclusion, several metabolic pathways involved in cellular and embryonic signalization of different biological processes affect embryo post-criopreservation survival.

Key Words: cryopreservation, lipid composition, lipid metabolism, lipidome, MALDI-MS, mass spectrometry, transcriptome, microarray.

1- INTRODUÇÃO

O Brasil possui o maior rebanho bovino comercial do mundo, ultrapassando 205 milhões de cabeças (IBGE). Neste contexto, estudos sobre biotecnologias capazes de aperfeiçoar a produção estão em franca ascensão, principalmente no que se refere às biotécnicas da reprodução.

Dentre as biotecnologias reprodutivas, atualmente a produção *in vitro* (PIV) de embriões está em evidência mundial. Fato este, em grande parte, atribuído ao sucesso da aplicação comercial brasileira. O país é líder mundial e referência na PIV de embriões bovinos. Em 2011, realizou cerca de 318 mil transferências de embriões bovinos PIV, o que equivale a 85% de toda movimentação mundial (STROUD, 2012; VIANA 2012). Grande parcela desse sucesso é devido ao elevado número de oócitos obtidos por aspiração folicular guiada por ultrassonografia em doadoras de raças zebuínas, principalmente representada pela raça Nelore (*Bos taurus indicus*), o que possibilita sua aplicação comercial em programas de larga escala (PONTES et al., 2011).

Porém, apesar dos números extremamente favoráveis da PIV, acompanhado da transferência desses embriões a “fresco”, a aplicação da criopreservação embrionária encontra-se extremamente limitada. A despeito de alguns avanços na criopreservação dos embriões PIV nas últimas duas décadas, a criopreservação se mantém como uma das áreas mais desafiadoras das tecnologias embrionárias uma vez que ainda não apresenta consistência de resultados, o que reflete diretamente na baixa porcentagem de embriões PIV criopreservados e transferidos em relação ao total transferido no Brasil (5 – 7 %) e no mundo (7 - 8 %) nos últimos anos (STROUD, 2010; VIANA et al*.,* 2010; STROUD, 2012; VIANA, 2012).

A criopreservação é uma tecnologia de reprodução assistida que possibilita o armazenamento de embriões excedentes oriundos de programas de PIV e produção *in vivo* (TE), permitindo ser comercializado ou transferido no momento mais conveniente possível. É considerada uma estratégia fundamental para superar alguns desafios logísticos inerentes a transferência de uma grande quantidade de embriões frescos e principalmente para a viabilização do comércio de embriões bovinos para mercados

internacionais, particularmente pela posição do país como referência em genética bovina adaptada aos trópicos. Entretanto, os resultados modestos da criopreservação dos embriões PIV limitam a sua aplicação na rotina do manejo reprodutivo de fazendas de forma semelhante ao que é feito com o sêmen na inseminação artificial, o que exigirá um grande esforço da pesquisa científica para superar esses desafios .

Desde o primeiro sucesso na criopreservação de embriões de mamíferos (WHITTINGHAM et al., 1972), é conhecido que os embriões advindos de sistemas de PIV são mais sensíveis à criopreservação do que embriões TE (LEIBO and LOSKUTOFF, 1993). Tal fato pode estar relacionado a algumas diferenças entre os embriões provenientes das duas técnicas. Dentre estas, o grande número de gotas lipídicas citoplasmáticas observadas nos embriões PIV vem sendo apontado como a principal causa da sua reduzida criotolerância quando comparada aos embriões TE (ABE et al., 2002). Devido a este fato, o estudo do conteúdo e da composição lipídica dos embriões se tornou um dos principais focos da pesquisa na área da criopreservação embrionária.

Ainda é desconhecido de que forma o acúmulo lipídico ocorre no citoplasma celular embrionário. Estudos comprovaram que o meio de cultivo embrionário é um fator determinante na qualidade dos blastocistos (RIZOS et al., 2003; SUDANO et al., 2011), e que o aumento do número de gotas lipídicas citoplasmáticas pode ocorrer como resultado da suplementação do meio de cultivo com soro fetal bovino (SFB) (ABE et al., 2002; RIZOS et al., 2002; SUDANO et al., 2011), ou devido a anormalidades no metabolismo energético embrionário, incluindo o “Crabtree effect” (DE LA TORRE-SANCHEZ et al*.,* 2006).

Neste contexto de acúmulo lipídico embrionário, a introdução de agentes químicos visando modular o acúmulo lipídico através da atuação sobre as vias llipogênicas/lipolíticas, como por exemplo, o etossulfato de fenazina (SUDANO et al., 2011), o forskolin (PASCHOAL et al., 2012), e a L-carnitina (SUTTON-MCDOWALL et al., 2012; TAKAHASHI et al., 2012); e a implementação de meios definidos sem o uso do SFB (ABE et al., 2002; MUCCI et al*.,* 2006), estão sendo preconizados para tornar o embrião PIV mais resistente as técnicas de criopreservação.

Apesar de todo envolvimento das gotas lipídicas citoplasmáticas na elevada sensibilidade dos embriões PIV frente a criopreservação, em um estudo descritivo da ultraestrutura de embriões taurinos e zebuínos TE, constou-se que os embriões taurinos possuem maior acúmulo lipídico do que os zebuínos, porém, são os embriões taurinos que se mostraram mais resistentes ao processo de criopreservação *(VISINTIN* et al., 2002). Recentemente, foi descrito que a despeito do aumento do acúmulo lipídico estar diretamente associado com a redução da criotolerância, a qualidade dos embriões representados pela porcentagem de células em apoptose prediz melhor a sua capacidade de sobreviver após a criopreservação (SUDANO et al., 2011; Sudano et al., 2012). Esses resultados sugerem que o estudo da real contribuição do conteúdo lipídico dos embriões sobre a criotolerância deve ser expandido para a composição lipídica embrionária o que é crucial para o sucesso da criopreservação.

Sabe-se que os triglicerídeos são os lipídeos predominantes presentes no citoplasma das células dos mamíferos na forma de gotas lipídicas (MCKEEGAN and STURMEY, 2011) constituindo uma importante fonte energética para os oócitos e embriões (STURMEY et al., 2009). Por outro lado, os fosfolipídeos são os lipídeos mais abundantes na membrana celular dos organismos eucariontes, e o seu papel para o sucesso da criopreservação ainda é pouco conhecido (VAN MEER et al., 2008). Os fosfolipídeos, particularmente as fosfatidilcolinas (PC) e as esfingomielinas (SM), são as principais unidades funcionais das membranas, e as suas composições determinam as propriedades físico-químicas das membranas celulares incluindo fluidez, permeabilidade, e o comportamento as mudanças de temperatura (EDIDIN, 2003).

Tendo em vista aumentar a fluidez de membrana dos embriões, diversos estudos vem sendo conduzidos na tentativa de aumentar o grau de insaturação dos lipídeos da membrana celular através da incorporação de ácidos graxos polinsaturados. Uma das abordagens seria a suplementação dos ácidos graxos polinsaturados diretamente no meio de cultivo embrionário, como no caso do uso do acido linoléico-albumina sérica bovina em estudo conduzido com o congelamento convencional (HOCHI et al., 1999) assim como o do acido linoléico conjugado no caso da vitrificação (PEREIRA et al.,

2007; PEREIRA et al., 2008) de embriões bovinos PIV. Outra abordagem seria através do manejo nutricional das doadoras fornecendo uma dieta rica em ácidos graxos polinsaturados visando a incorporação do mesmo na membrana celular de oócitos (ZERON et al., 2002) e embriões (KOJIMA et al., 1996).

Esforços para o entendimento do metabolismo embrionário vem sendo conduzidos para o esclarecimento das vias bioquímicas envolvidas no acúmulo e na composição lipídica. Dentre estes, encontra-se o estudo do padrão de expressão de genes envolvidos no metabolismo lipídico (AL DARWICH et al., 2010; MCKEEGAN and STURMEY, 2011). Além da tentativa de melhorar a composição lipídica, a qualidade embrionária é outro ponto chave da capacidade dos embriões em sobreviver após a criopreservação. De fato, um elevado grau de comprometimento das células embrionárias resultam em uma reduzida criotolerância (SUDANO et al*.,* 2012).

Frequentemente, a morfologia embrionária e as taxas de clivagem e de blastocistos são critérios utilizados para avaliação da competência embrionária (Alikani *et al.,* 2002). Entretanto, com o advento de novas biotecnologias, tem-se tornado claro que a competência embrionária pode ser severamente comprometida sem alterações morfológicas perceptíveis. A viabilidade do embrião pode ser mensurada através de outras maneiras, tais como a apoptose, criotolerância, e a partir de ferramentas de biologia molecular (WRENZYCKI et al., 2004; WRENZYCKI et al., 2005).

Já foi descrito que o padrão de expressão gênica pode ser utilizado como marcador de qualidade embrionária, efetividade do meio de cultivo, metabolismo lipídico e habilidade do embrião em gerar prenhez (AL DARWICH et al., 2010; KUZMANY et al., 2011; STINSHOFF et al., 2011). Além disso, uma expressão elevada de um grande número de genes já foi observada em embriões PIV quando comparada aos embriões TE (CORCORAN et al., 2006; CÔTÉ et al., 2011), o que provavelmente está associado a um metabolismo exacerbado (“unquite metabolism”) em função da exposição dos embriões a um ambiente de estresse favorecendo a geração das espécies reativas de oxigênio, e consequentemente, estresse oxidativo (LEESE et al., 2008). Com o advento das tecnologias de biotecnologia molecular de alto rendimento, o padrão de expressão gênica global vem sendo utilizado como um ferramenta muito

poderosa para o estudo simultâneo da abundância de diferentes transcritos em diversas áreas das tecnologias da reprodução assistida (GAD et al., 2012).

Portanto, o presente estudo teve como objetivo avaliar a criotolerância, o conteúdo lipídico, a composição lipídica e o padrão de expressão gênica global de blastocistos *Bos taurus indicus* e *Bos taurus taurus* produzidos *in vitro* e *in vivo*. Para tanto, um arranjo fatorial 2 x 2, com duas subespécies (*Bos taurus indicus* vs *Bos taurus taurus*) e duas origens (PIV vs TE) será utilizado para testar a hipótese de que a capacidade de sobrevivência após a criopreservação é um evento multifatorial, e que a composição lipídica e o padrão de expressão gênica global afetam a criotolerância embrionária.

2- CAPÍTULO I

PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN PROFILES VARY IN BOS TAURUS INDICUS AND BOS TAURUS TAURUS IN VITRO- AND IN VIVO-PRODUCED BLASTOCYSTS

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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]

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Short title:
Lipids of Nellore and Simmental Embryos

Summary sentence
Taurine and IVP embryos had more lipid than zebuine and ET. However, taurine embryos had higher cryotolerance than zebuine. Through MALDI-MS, different lipid profiles were obtained, and possible embryo cryotolerance biomarkers were established.

Key words: Lipidomics, zebuine, taurine, embryo, mass spectrometry, in vitro culture, MALDI-MS, cryotolerance biomarkers

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 on differences in membrane lipids in embryo, such as phosphatidylcholines (PC) and sphingomyelins (SM). The objective of this work was to compare the profiles of several PC and SM species and relate this information to cytoplasmic lipid levels present in Nellore (*Bos taurus indicus*) and Simmental (*Bos 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 compared to ET-derived embryos. Therefore, mass spectrometry profile 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 versus 34:2), as were IVP- and ET-derived embryos (32:0 versus 36:5), they are potential PC markers of post-cryopreservation embryonic survival.

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 embryo IVP over past decades, cryopreservation is still 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 in vivo-

derived embryos and as a result have reduced post-cryopreservation 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 cryopreservation success is still poorly understood [16]. Phospholipids, most particularly phosphatidylcholines (PC) and sphingomyelins (SM), are structural units of functional membranes, and their composition determines most physico-chemical 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 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 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 (*Bos taurus indicus* and *Bos taurus taurus*) and origin (IVP and ET), suggesting that some PC species might be used as biomarkers of the higher post-cryopreservation survival observed in Simmental and ET embryos.

MATERIAL AND METHODS

Experimental Design

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

Reagents Used

All materials were acquired from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA), except when specified.

Weather, Animals and Feed

The local weather is tropical (CwA Köppen's classification), characterized by a rainy, hot summer and a dry winter. Non-lactating mature Nellore (*Bos taurus indicus*) (n=21; 4 to 10 years old; 446±18 kg body weight) and Simmental (*Bos taurus taurus*) (n=22; 3 to 6 years old; 498±21 kg body weight) cows were used in this study. All animals were considered 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 (OPU) procedures were carried out on visible follicles ≥ 2 mm in diameter using a B-mode ultrasound scanner (Mindray® Bio-medical Eletronics Co, Ltd., Shenzhen, China), equipped with a 7.5 MHz convex array transducer fitted into the intravaginal device (Watanabe®, Ltd., Cravinhos, SP, Brazil), and a stainless steel guide. Follicular puncture was performed using a disposable 19-gauge, 3 1/2-inch hypodermic needle (Becton® Dickinson, Curitiba, PR, Brazil) connected to a 50 mL conical tube (Corning®, Acton, MA, USA) 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 phosphate-buffered saline solution (PBS, Nutricell, Campinas, SP, Brazil) supplemented with 1 mL/L heparin (Hemofol®, Cristalia, Belo Horizonte, BH, Brazil).

In Vitro Maturation (IVM)

The aspirated content was immediately filtered, and oocytes were washed three times in TCM 199 with Earle's salts (Gibco®, Invitrogen Co., Grand Island, NY, USA) supplemented with 25 mM HEPES, 10% FCS, 100 μg/mL of streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co., Grand Island, NY, USA). 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 ^{0}C in 5% CO_2 in air with saturated humidity for 24 h. Drops containing 90 μL of TCM 199 with Earle's salts and L-glutamine (Gibco®, Invitrogen Co., Grand Island, NY, USA) supplemented with 5 mg/mL BSA (fatty acid free, Sigma A-8806), 0.2 mM sodium pyruvate, 5 mg/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada), 1 mg/mL FSH (Folltropin®, Bioniche Co., Belleville, ON, Canada), 100 μg/mL of streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co.), containing 20-30 oocytes each, were placed in petri dishes and covered with mineral oil.

In Vitro Fertilization (IVF)

At the end of the maturation period, groups of 20-30 oocytes were transferred to 90 μL-containing drops of fertilization media covered with mineral oil. Nellore- and Simmental-derived oocytes were subjected to 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 x 10^6 sperm/mL. Fertilization occurred in HTF (Irvine Scientific Co., Santa Ana, CA, USA) supplemented with 5 mg/mL BSA (fatty-acid-free, Sigma A-8806), 0.2 mM pyruvate, 30 μg/mL heparin, 18 μM penicilamine, 10 μM hypotaurine, 1.8 μM epinephrine, 100 μg/mL streptomycin sulfate, and 100 IU/mL penicillin (Gibco®, Invitrogen Co.). Oocytes and sperm were incubated under the same conditions as IVM for approximately 18 h. The day of fertilization was defined as Day 0.

In Vitro Culture (IVC)

Eighteen hours post-IVF, presumptive zygotes were denuded by repeated pipetting and transferred to culture plates in drops containing 90 μL of culture media (20-30 structures per 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 SOFaa [20] with 2.7 mM myo-inosytol, 0.2 mM pyruvate, low FCS supplementation (2.5% - to avoid deleterious effect as described previously [3] since FCS is a protein supplement added in most culture media used for bovine embryo research and commercial production), 5 mg/mL BSA (fatty-acid-free, Sigma A-8806), 100 μg/mL streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co.). 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, non-lactating Nellore (*Bos taurus indicus*, N=7) and Simmental (*Bos taurus taurus*, N=8) cows with good body condition scores. The ovarian superstimulation protocol was based on that used in [21]. On Day 0 (without regard to the stage of the estrous cycle), cows received an intra-vaginal insert containing 1.9 g progesterone (CIDR®, Pfizer Animal Health, São Paulo, SP, Brazil) and 2 mg of estradiol benzoate i.m. (Estrogin®, Farmavet Co., São Paulo, SP, Brazil). Starting on Day 4, cows received 200 mg of FSHp (Folltropin-V®, Bioniche Co.) 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 Co.) 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®, Bioniche Co.) 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 N= 39, respectively, for Nellore and Simmental cows) was carried out through uterine flushing with a phosphate-buffered saline solution (PBS, Nutricell Co.) 7.5 d after the first AI, as previously described [22].

Semi-Quantitative Lipid Assay

A sample of blastocysts (n=15 /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% PVA in distilled water. Prepared embryos were mounted in 10 μL glycerol on cover slips and examined under a light microscope at 600x 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, USA). Embryo color pictures were converted to a gray scale image (Fig. 1) and embryos were delimited to obtain area (μm^2) and gray intensity mean (arbitrary unit) 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 unit / 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-derived embryo was washed five times in drops of phosphate-buffered saline solution (PBS) 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 solution of 1:1 (v/v) methanol/ultrapure water (ACS/HPLC grade; Burdick and Jackson, Muskegon, MI, USA and Millipore, Bedford, MA, USA) 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 location was recorded. Just before analysis, 1 μL of 1.0 M of 2,5-dihydroxybenzoic acid (DHB) 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, Bremen, Germany) equipped with smart beam laser technology. The MS data were acquired in the 700-1200 *m/z* range by averaging 1,500 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 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 considered from each spectrum and used as the starting point to search for *m/z* values corresponding to lipids.

Lipid Assignment

MALDI-MS/MS (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 or www.lipidmaps.org) were utilized to assign phospholipid 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 Inst. Inc., Cary, NC, USA). Sources of variation in the model included subspecies (*Bos taurus indicus* - Nellore vs *Bos taurus taurus* - Simmental), origin (IVP versus ET) and first-order interactions; all factors were considered fixed effects. If the ANOVA was significant, means were separated using Tukey's 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 mass spectrometry profiles, multivariate and univariate statistical models were used. A first principal component analysis (PCA) was performed using Pirouette v.3.11 (Infometrix Inc., Woodinville, WA, USA) 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 generalized linear mixed model (GLIMMIX) procedure with SAS software to better study the significance of each ion for all groups. Sources of variation in the model included subspecies (*Bos taurus indicus* - Nellore versus *Bos taurus taurus* - Simmental), origin (IVP vs ET) and first-order interactions; all factors were considered 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 did 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 there was a random distribution of lipid droplets 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, and separated by a colon. Phospholipid 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 or www.lipidmaps.org). Fig. 3 displays a representative lipid profile of each group. More than one isomer can occur for a single lipid.

PCA analysis shows that the four experimental groups can be resolved via their MALDI-MS profiles because the 2D-PCA plots cluster samples corresponding to different

breeds (Nellore versus Simmental) and origins (IVP versus ET), with little overlap between Nellore and Simmental IVP groups (Fig. 4A).

However, in the 3D-PCA plot, 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 analysis 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, 760.6, corresponding to protonated PC (32:1), PC (34:2) and PC (34:1), respectively. PC (32:1) and PC (34:1) presented higher abundance ($P<0.05$) in Nellore embryos, while 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, and corresponding to protonated PC (32:1), PC (34:2) and sodiated PC (36:5). The abundance of these three lipid ions was elevated ($P<0.05$) in ET-derived embryos when 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. The SM (16:0) and PC (36:2) ion abundances were increased ($P<0.05$) in Simmental ET embryos, while 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 when compared with IVP. Nellore ET embryos also had a low abundance of SM (16:0) compared with Simmental IVP (Fig. 6).

Nellore ET-derived 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 than in Simmental IVP embryos (Fig. 6).

MS/MS Characterization of Phospholipids 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 dihydrogenphosphate choline ($C_5H_{15}PO_4N$) (Fig. 7).

DISCUSSION

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

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 inter-cellular transport [28, 29]. A series of studies on lipid composition in bovine embryos has examined fatty acid triglyceride and lipid content composition [4-6, 11, 30]. Lower post-cryopreservation embryo survival has also frequently been associated with high embryo lipid content [4, 9, 10].

It is still not clear why cytoplasmic lipid accumulation affects embryonic cryotolerance; controversy persists in the literature. In an ultrastructural descriptive study, while ET taurine (*Bos taurus taurus*) embryos presented greater cytoplasmic lipid accumulation than zebuine embryos, the former showed higher cryotolerance [31]. Arreseigor *et al*. [32] reported that

taurine frozen ET embryos resulted in a higher pregnancy rate than zebuine. 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 cryopreservation success.

Preliminary data showed that Simmental embryos had higher post-vitrification survival when compared with Nellore embryos (34.6 *vs*. 20.2 % of hatching/hatched; P= 0.04; respectively), and ET embryos had higher survival rate after vitrification than IVP (38.5 *vs.* 18.1% of hatching/hatched; P= 0.004; respectively) [34]. These data can be correlated with our Sudan black B staining data, which corroborates previous work [3, 5, 11, 31] indicating that *Bos taurus taurus* embryos have higher lipid content than *Bos taurus indicus* and that IVP (which involves in vitro culture) leads to more cytoplasmic lipid than ET methods. In summary, the origin effect (IVP versus 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, 35, 36]. In the present experiment, the majority of lipid droplets in ET blastocysts occurred in the ICM, not 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 serve as 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 address the difficulties of post-cryopreservation embryo survival. Changes in the lipid structural composition of the membranes, which are difficult to detect using staining procedures, are readily detected by mass spectrometry. These changes are associated with the impact of in vitro culture conditions, and especially with FCS supplementation in the culture media, and result in negative effects on embryo survival after cryopreservation that have previously been reported [3, 4, 6, 10, 33].

MALDI-MS has proven to be a powerful tool in lipidomics [43], generating PL (mainly PC and SM in the positive ion mode) and TAG profiles with simple interpretation. Previously work by our group described a practical and novel approach for direct 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 versus FCS) and atmospheric pressure (5% versus 20%) conditions [18].

In the present work, 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, there was some overlap between Simmental and Nellore IVP-derived 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 had a low relevance for embryo post-cryopreservation survival at the present work, this PL play an important role in the heat-shock-induced apoptosis through sphingomyelin pathway associated with the generation of ceramide [47]. However, higher membrane fluidity was expected with higher unsaturated lipid content favoring post-cryopreservation 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 post-cryopreservation 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], while 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 phospholipid composition, length of fatty acyl residues, number and position of the double bounds within phospholipids, the ratio of saturated to unsaturated fatty acid total content, cholesterol level, and the protein content [28, 53-55]. The change or the imbalance of one or more of these factors could lead to different rates of cryopreservation success. In the present work, we have observed variations of unsaturation degree of phospholipids fatty acyl residues and also differences in phospholipid 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 bounds in the membrane lipids increase membrane fluidity in lower ambient temperatures. The fact that *Bos taurus taurus* have been naturally selected in Europe under cold temperatures might have determined the higher degree of unsaturated phospholipid present in Simmental blastocysts.

In conclusion, a semi-quantitative analysis of cytoplasmic lipids using Sudan black B staining showed that Simmental embryos had a higher cytoplasmic lipid content than Nellore embryos, and IVP-derived embryos have more cytoplasmic lipids 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 post-cryopreservation survival of bovine blastocysts.

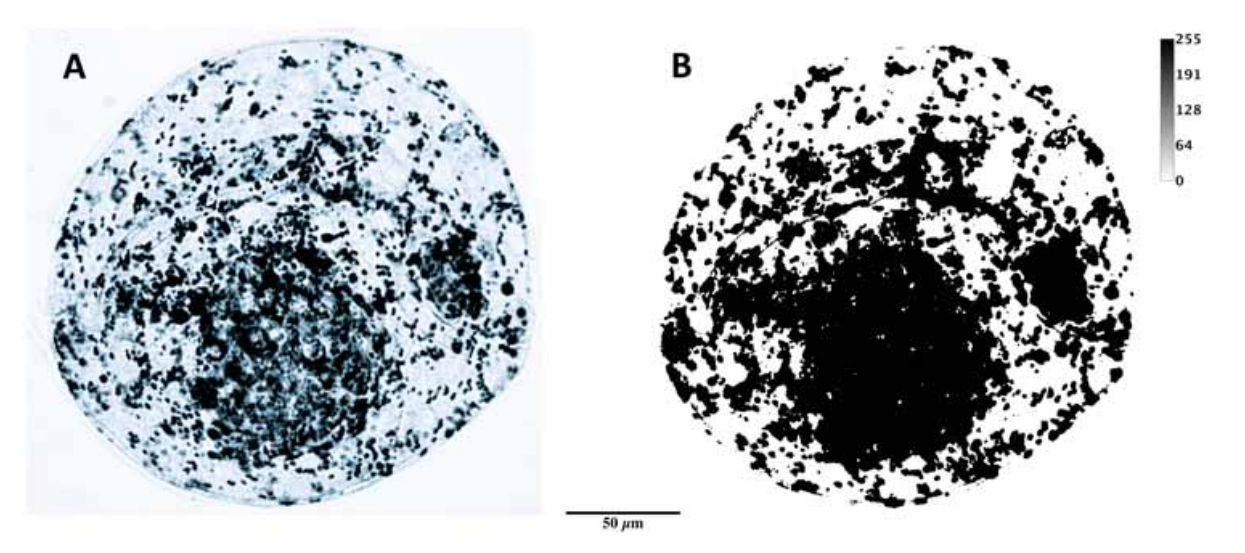


FIG. 1. Optical view of an embryo stained with Sudan black B (**A**) and gray-scale conversion for semiquantitative lipid content assay (**B**). Original magnification x 600.

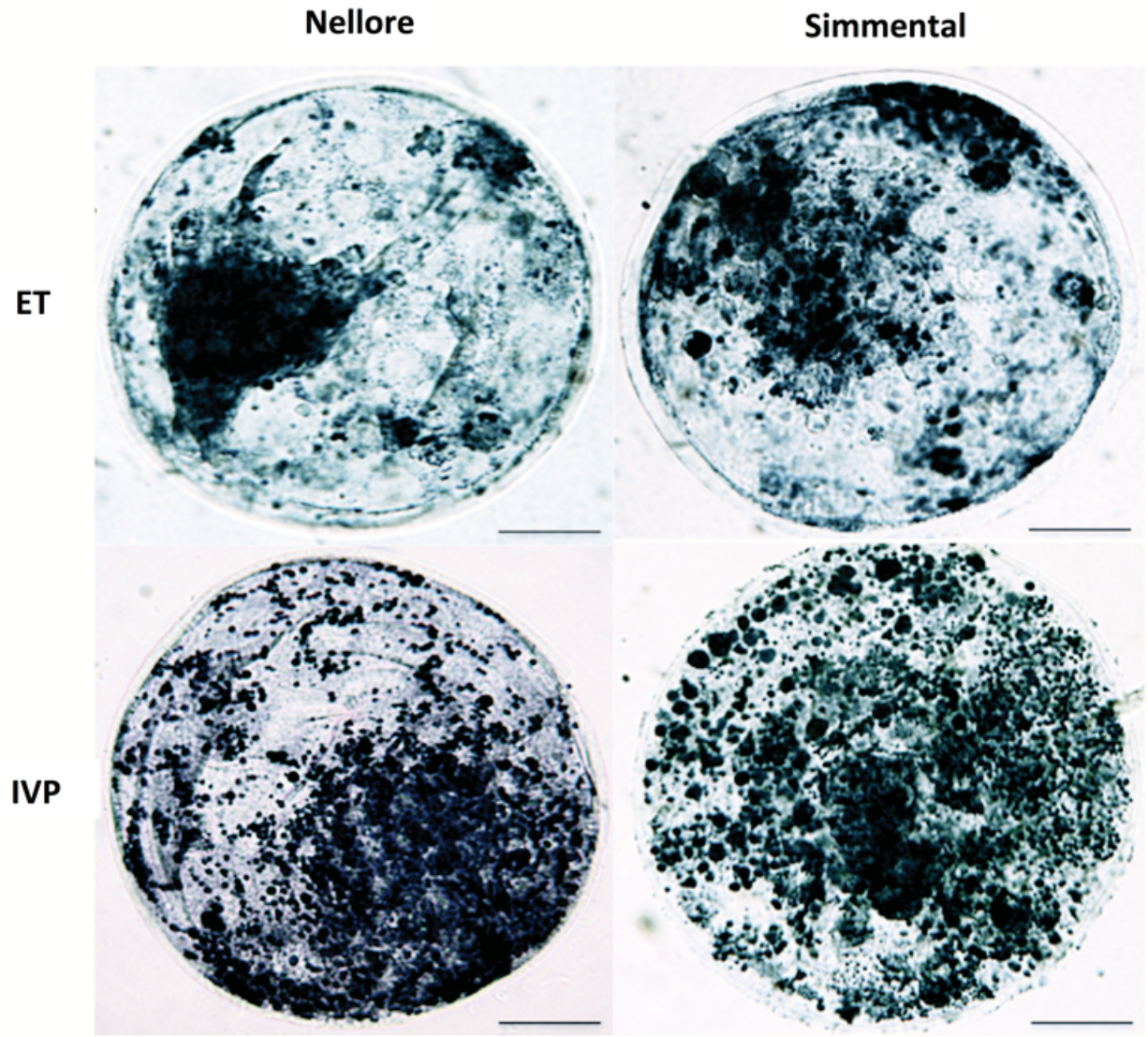


FIG. 2. Light micrographs of Nellore and Simmental ET and IVP embryos stained with Sudan black B. Black areas indicate sudanophilic cytoplasmic lipid droplets. Original magnification x 600; bar = 50 μm.

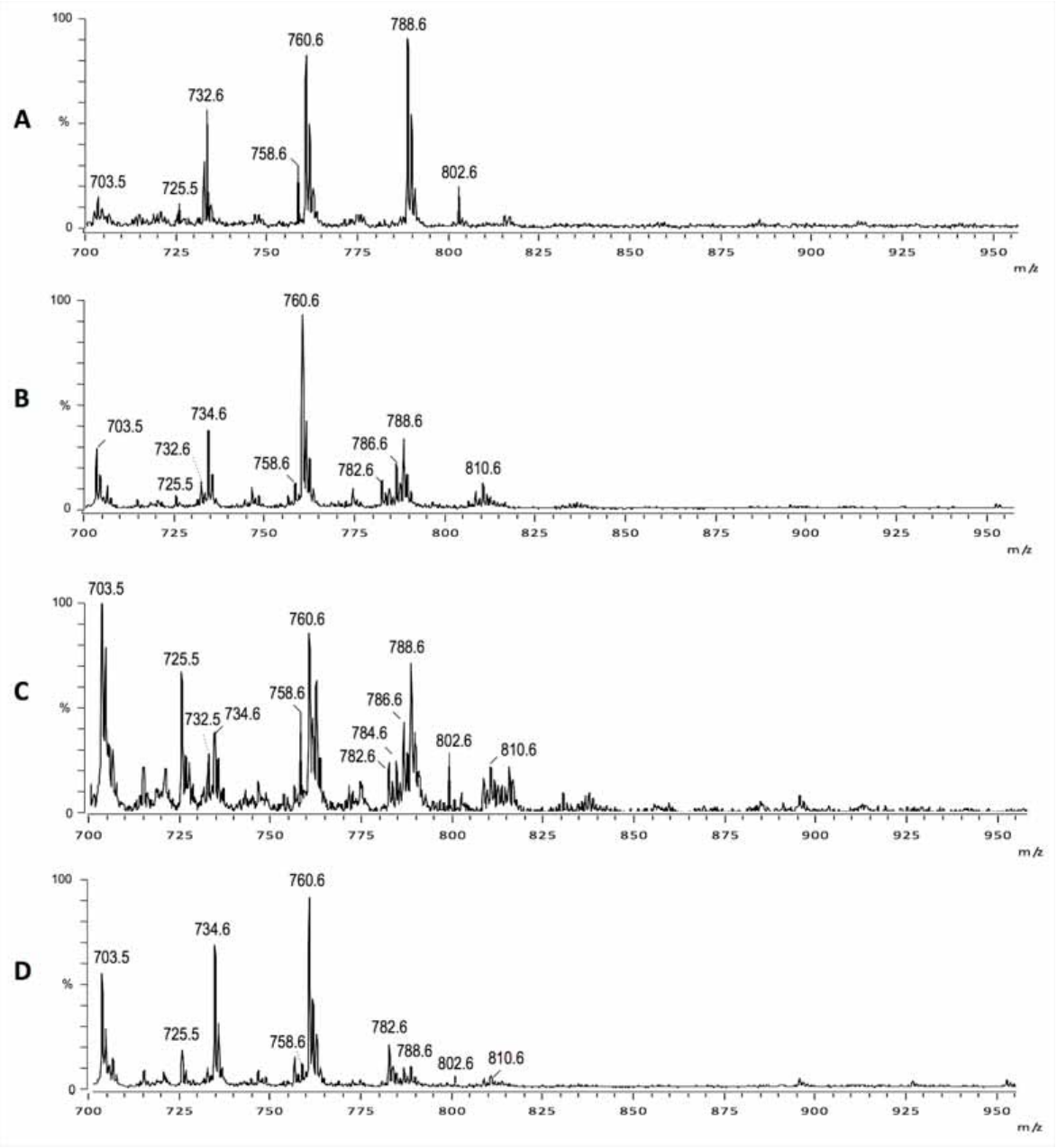


FIG. 3. Representative MALDI-MS in the positive-ion mode for Nellore and Simmental ET and IVP individual embryos. **A**) Nellore ET embryo. **B**) Nellore IVP embryo. **C**) Simmental ET embryo. **D**) Simmental IVP embryo.

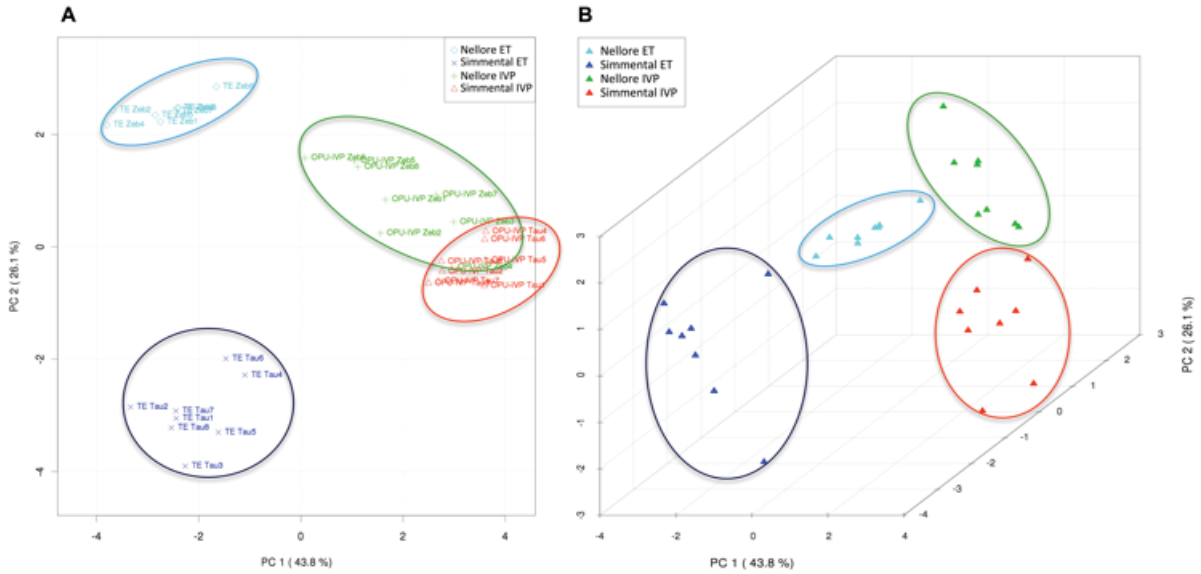


FIG. 4. Two-dimensional (**A**) and three-dimensional (**B**) PCA plots for MALDI-MS data of Nellore and Simmental ET and IVP embryos (n = 8 per group).

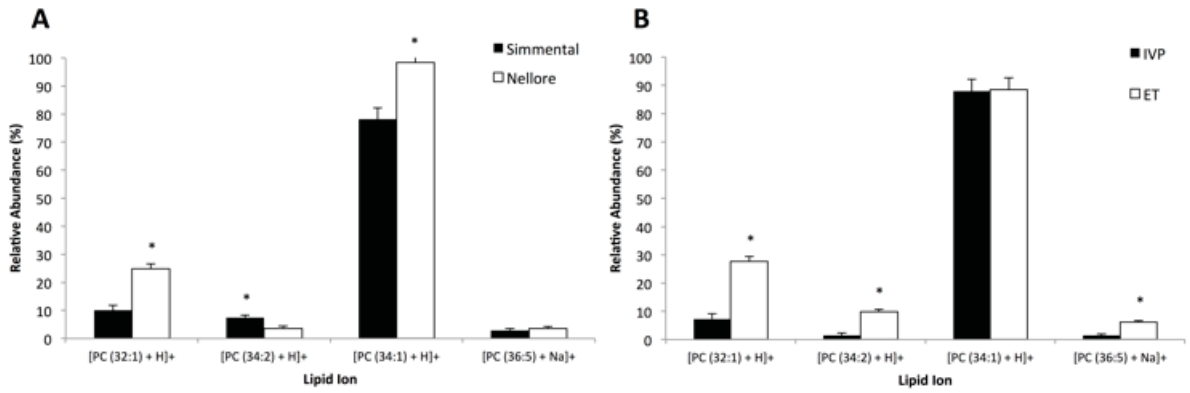


FIG. 5. Subspecies (**A**) and origin (**B**) effects on the relative intensity of lipid ions presented in the Nellore and Simmental IVP and ET embryos (leastsquares mean ± SEM). Asterisk indicates differences between pairs of dark and light bars ($P < 0.05$, n = 16 per group).

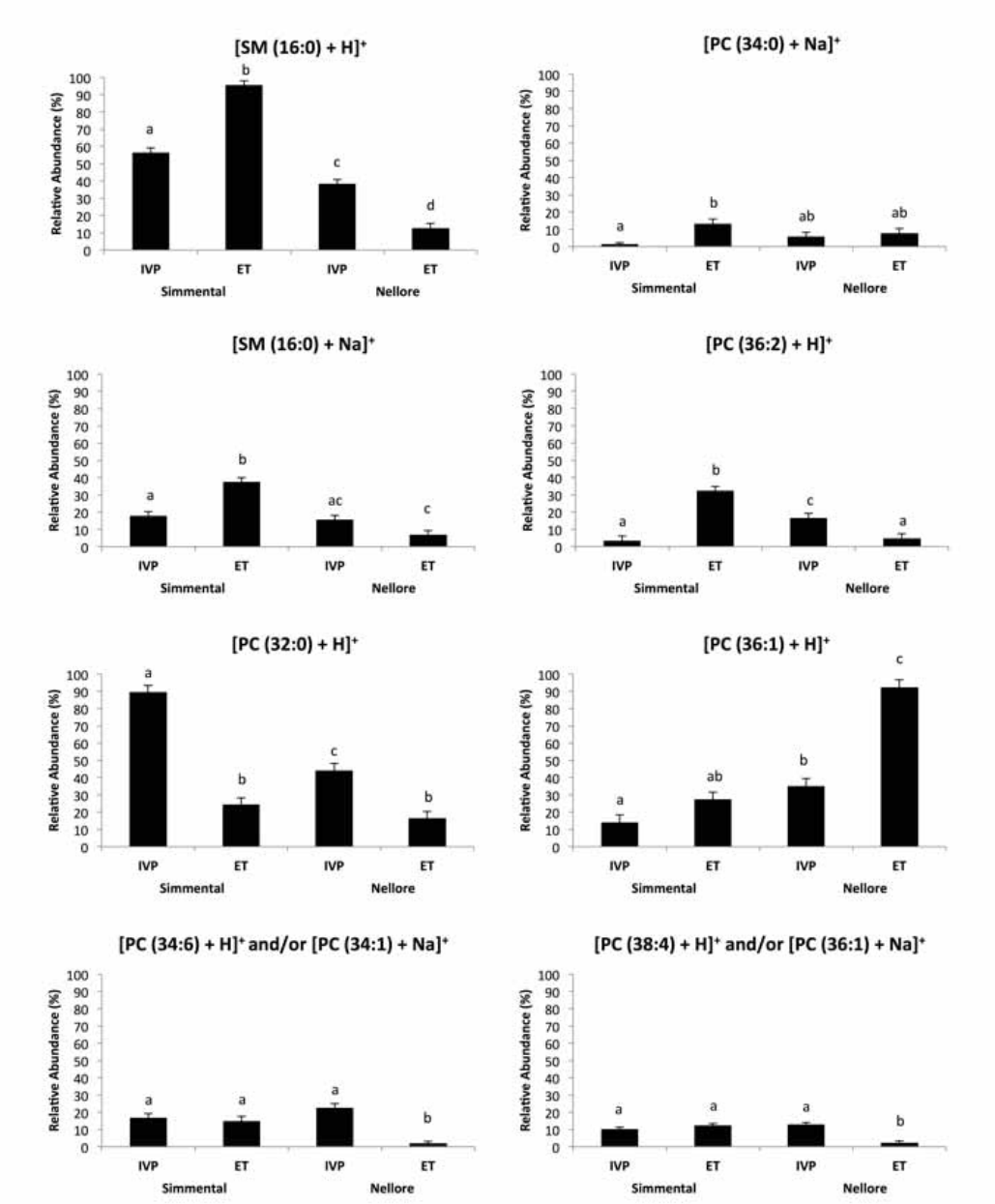


FIG. 6. Relative abundance of lipid ions present in Nellore and Simmental IVP and ET embryos. Values without common lowercase letters differ significantly ($P < 0.05$, $n = 8$ per group).

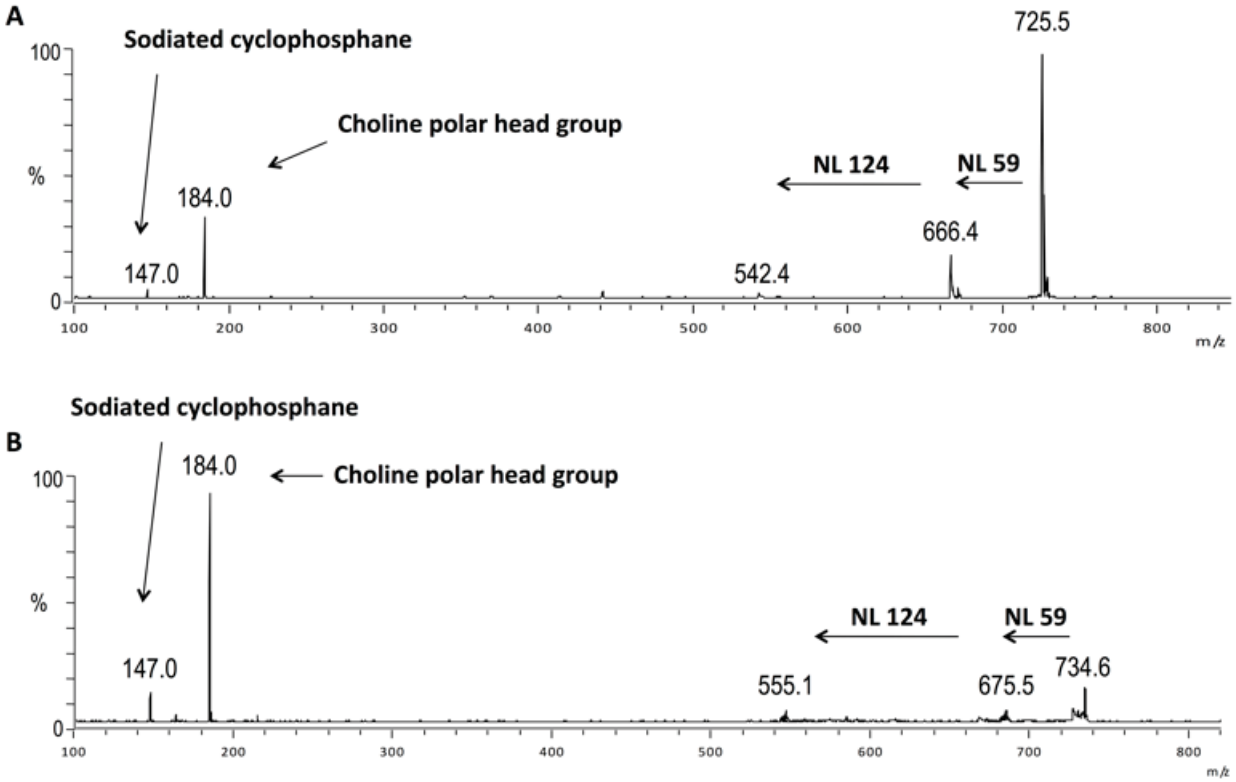
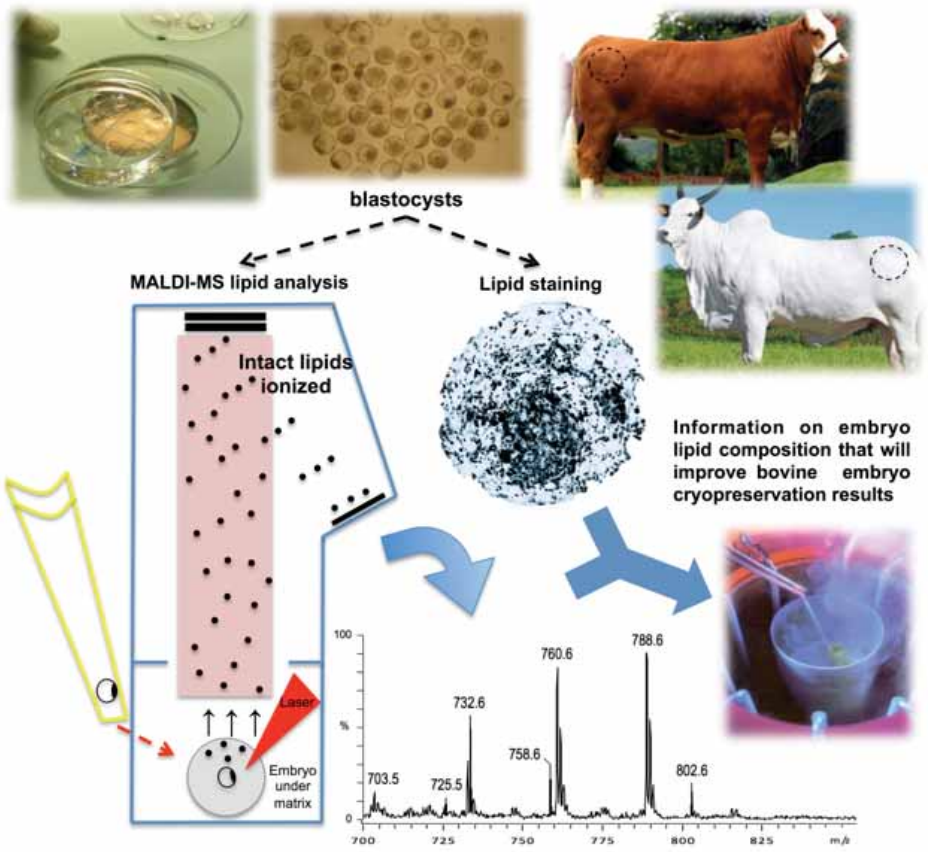


FIG. 7. MALDI-MS/MS for **m**/**z** 725.5 $[SM (16:0) + Na]^+$ (**A**) and **m**/**z** 734.6 $[PC (32:0) + H]^+$ (**B**). Both spectra display characteristic neutral loss (NL) of 59 Da (trimethylamine) and of 124 Da (cyclophosphane ring). Sodiated cyclophosphane (**m**/**z** 147.0) and choline polar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



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

A	*m/z*	Lipid Ion (carbons:unsaturation)	Subspecie			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.

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).

Parameter	Subspecies			Origin		
	Bos indicus (Nellore)	*Bos 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 / area (x $10^{-4}/\mu m^2$)	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 / volume (x $10^{-4}/\mu m^3$)	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 (P<0.05).
[A,B] Within a row, origin means without a common superscript differ (P<0.05).
N= 30 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).

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3- CAPÍTULO II

CRYOTOLERANCE AND GLOBAL GENE EXPRESSION PATTERNS OF *BOS TAURUS INDICUS* AND *BOS TAURUS TAURUS IN VITRO-* AND *IN VIVO*-PRODUCED BLASTOCYSTS

O artigo foi submetido ao periódico Reproduction e encontra-se de acordo com as normas de submissão exigidas.

Title:

Cryotolerance and global gene expression patterns of *Bos taurus indicus* and *Bos taurus taurus in vitro*- and *in vivo*-produced blastocysts

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Short title

Cryotolerance of Nellore and Simmental blastocysts

Abstract

Embryo quality, *in vitro* culture conditions and lipid composition are known to influence the embryo postcryopreservation survival. Limited information is available about the relationship between embryo gene expression, lipid metabolism/composition and cryotolerance. In this study, we assessed the cryotolerance and global gene expression patterns in Nellore (*Bos taurus indicus*) and Simmental (*Bos taurus taurus*) blastocysts produced *in vitro* (IVP) and *in vivo* (ET). Blastocyst production was higher in Nellore than in Simmental cows. The total

numbers of ova/embryos recovered and transferable embryos were not different between the breeds. Simmental and ET (34.6% and 38.5%, N= 75 and 70) blastocysts had higher survival rates after cryopreservation compared to Nellore and IVP (20.2% and 18.1%, N= 89 and 94) embryos, respectively. Differences between transcriptomes were addressed by principal component analysis, which indicated that gene expression was affected by subspecies (158 genes), origin (532 genes), and interaction between both subspecies and origin (53 genes). Differentially expressed genes involved in the free radical scavenging, lipid and acid nucleic metabolism, and cell death and survival were identified. The up-regulation of *AUH* and *ELOVL6* in Simmental and of *ACSL3* and *ACSL6* in IVP embryos could explain different lipid compositions and postcryopreservation survival capacities. The expression profiles of genes related to mitochondrial metabolism (*ATP5B*), oxidative stress (*GPX4*), apoptosis (*DAD1*, *DAP*, *PRDX2*), heat shock (*HSPA5*), pregnancy (*IFNT2*, *PAG2*), and cell differentiation (*KRT18*) differed between subspecies and origin and could elucidate the different levels of embryo viability and cryotolerance.

Introduction

The *in vitro* production of *B. taurus indicus* (Zebu breeds) embryos is performed commercially at a large scale due to the higher number of oocytes obtained by ultrasound-guided follicular aspiration in these breeds (Pontes, et al. 2011). The cryopreservation of bovine embryos is important for overcoming the logistical challenges inherent in the transfer of large numbers of fresh embryos and for expanding the commercialization of embryos between countries. Embryos produced *in vitro* are more sensitive to cryopreservation than those produced *in vivo* after ovarian superstimulation. Despite the advances achieved over the last two decades, cryopreservation is still one of the most challenging areas in embryo technology (Saragusty and Arav 2011, Sudano, et al. 2011b) and could be an interesting model for researching embryo biology after exposures to supraphysiologic stress (Sudano, et al. 2012a).

The large amount of cytoplasmic lipid droplets observed in IVP embryos has been suggested to be the major cause of reduced postcryopreservation survival (Abe, et al. 2002). Triglycerides are the predominant lipid in the cytoplasm of mammalian cells, which is where they form lipid droplets (McKeegan and Sturmey 2011). These stored lipids constitute an important source of energy for oocytes and embryos

(Sturmey, et al. 2009). On the other hand, phospholipids (PL) are the most abundant lipid in eukaryotic cell membranes, and their role in successful cryopreservation remains poorly understood (van Meer, et al. 2008). Phospholipids, particularly phosphatidylcholines (PC) and sphingomyelins (SM), are structural units of functional membranes, and their composition determines the physicochemical properties of cell membranes, including fluidity, permeability, and thermal phase behavior (Edidin 2003). We have recently reported that the PL profiles of bovine embryos vary between subspecies (*B. taurus indicus* vs. *B. taurus taurus*) and origin (IVP vs. ET) and that lipid species can potentially be used as biomarkers of embryonic postcryopreservation survival. Our previous results have indicated that not only the lipid amount but also the lipid composition accounts for embryo survival after cryopreservation (Sudano, et al. 2012b).

Several studies have proposed that the mRNA expression levels of a variety of genes can be used as markers of the embryo quality, the effectiveness of the culture media, and the capacity to sustain a successful pregnancy (Kuzmany, et al. 2011, Stinshoff, et al. 2011). In fact, defects in a single gene have already been associated with embryonic implantation failure (Copp 1995). However, while analyses of global gene expression have proven valuable for improving embryo technologies, specific information on gene markers associated with embryo survival after cryopreservation is still lacking (Gad, et al. 2012, Sudano, et al. 2012a, Sudano, et al. 2012b).

In this study, we first compared the postcryopreservation survival of two different bovine subspecies and two embryo origins. Then, aiming to identify genes associated with embryo viability, lipid metabolism, and cryotolerance, we compared the global gene expression patterns of Nellore (*B. taurus indicus*) and Simmental (*B. taurus taurus*) blastocysts produced *in vitro* (IVP) and *in vivo* (ET). Considering our recent findings on different lipid profiles associated with subspecies and origin, we tested the hypothesis that cryotolerance and the expression levels of genes regulating lipid metabolism/composition, and embryo viability would be different between these groups.

Material 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

embryo origins—IVP and ET—on cryotolerance and global gene expression patterns. Single bovine embryos were used as the experimental unit in 8-94 replications.

Reagents used

All materials were acquired from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA), except when otherwise specified.

Weather, animals and feed

The local weather is tropical (CwA Köppen's classification), characterized by a rainy, hot summer and a dry winter. Nonlactating 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 sound after gynecological examination. The cows were managed under an intensive rotational grazing system based on highly productive artificially fertilized tropical pastures with free access to mineral supplements and water. The care and use of animal samples was approved by the Institutional Committee for Ethics in Animal Research of the São Paulo State University under protocol number 57/2010-CEUA.

In vitro production of Nellore and Simmental blastocysts by ultrasound-guided follicular aspiration

Ovum pick-up (OPU) procedures (N= 4 sessions) were performed on visible follicles ≥ 2 mm in diameter of Nellore (N = 14) and Simmental (N = 14) cows using a B-mode ultrasound scanner (Mindray® Bio-medical Electronics Co, Ltd., Shenzhen, China) equipped with a 7.5 MHz convex array transducer fitted into a proper intravaginal device (WTA Watanabe Tecnologia Aplicada®, Ltda., Cravinhos, SP, Brazil) and a stainless steel guide. Follicular puncture was performed using a disposable 19 gauge × 3 1/2-inch hypodermic needle (Becton® Dickinson, Curitiba, PR, Brazil) connected to a 50 mL conical tube (Corning®, Acton, MA, USA) via a silicon tube. Aspiration was performed using a vacuum pump (Watanabe, Ltda.) with a negative pressure of 10–12 mL water/min. The collection medium was phosphate-buffered saline solution (PBS, Nutricell, Campinas, SP, Brazil) supplemented with 1 mL/L heparin (Hemofol®, Cristália, Belo Horizonte, BH, Brazil).

In vitro maturation

The aspirated content was immediately filtered, and the oocytes were washed three times in TCM 199 with Earle's salts (Gibco®, Invitrogen Co., Grand Island, NY, USA) supplemented with 25 mM HEPES, 10% FCS (v/v), 100 μg/mL of streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co., Grand Island, NY, USA). Only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were used in the experiments. Selected oocytes were *in vitro* matured by incubation at 38.5ºC in 5% CO_2 in air with saturated humidity for 24 h. Drops containing 90 μL of TCM 199 with Earle's salts and L-glutamine (Gibco®, Invitrogen Co., Grand Island, NY, USA) supplemented with 5 mg/mL BSA (fatty acid-free), 0.2 mM sodium pyruvate, 5 mg/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada), 1 mg/mL FSH (Folltropin®, Bioniche Co., Belleville, ON, Canada), 100 μg/mL of streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co.) and 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 and covered with mineral oil. Nellore and Simmental oocytes were subjected to *in vitro* fertilization (IVF) with frozen semen from a single sample of each bull breed with proven fertility. Spermatozoa were selected using the Percoll method (Parrish, et al. 1995), and the concentration was adjusted to 2×10^6 sperm cells / mL. Fertilization occurred in human tubal fluid (HTF; Irvine Scientific Co., Santa Ana, CA, USA) supplemented with 5 mg/mL BSA (fatty acid-free), 0.2 mM pyruvate, 30 μg/mL heparin, 18 μM penicilamine, 10 μM hypotaurine, 1.8 μM epinephrine, 100 μg/mL streptomycin sulfate, and 100 IU/mL penicillin (Gibco®, Invitrogen Co.). Oocytes and sperm were incubated under the same conditions as the IVM procedure 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 (20–30 structures per drop), covered with mineral oil, and cultured in a humidified

atmosphere of 5% CO_2 and 5% O_2 with the balance N_2. The culture medium used was SOFaa (Holm, et al. 1999) supplemented with 2.7 mM myo-inosytol, 0.2 mM pyruvate, 2.5% FCS (v/v), 5 mg/mL BSA (fatty acid-free), 100 μg/mL streptomycin sulfate, and 100 IU/mL of penicillin (Gibco®, Invitrogen Co.). After 60 h of culture, cleavage was checked, and structures that were not cleaved were discarded. Embryos were transferred to 90 μL of the aforementioned culture media. The embryos remained in this condition until Day 7 when blastocyst production was evaluated. Blastocysts were vitrified (N = 94) or washed five times with PBS solution and stored at -80ºC in small volumes of PBS in 1.5 mL tubes until RNA extraction, which preceded the transcriptome analysis (a single blastocyst was used for each sample).

In vivo production of nellore and simmental blastocysts

Nellore (*B. taurus indicus*, N = 7) and Simmental (*B. taurus taurus*, N = 8) cows were submitted to the ovarian superstimulation protocol (N= 3 and 4 sessions, respectively, with 35 days apart) based on the procedure described by Barros and Nogueira (Barros and Nogueira 2001). On Day 0 (regardless of the stage of the estrous cycle), the cows received an intra-vaginal device containing 1.9 g progesterone (CIDR®, Pfizer Animal Health, São Paulo, SP, Brazil) and 2 mg of estradiol benzoate i.m. (Estrogin®, Farmavet Co., São Paulo, SP, Brazil). Starting on Day 4, the cows received 200 mg of porcine FSH (Folltropin-V®, Bioniche Co.) i.m. twice a day (equal concentration) in decreasing doses (40%, 30%, 20%, and 10%). On Day 6, two doses of 150 μg of sodium cloprostenol (Ciosin®, Intervet/Schering-Plough Animal Health Co.) were provided i.m. 12 h apart. The CIDR was removed 36 h after the first application of cloprostenol, and 12.5 mg of porcine LH (Lutropin-V®, Bioniche Co.) was given i.m. 48 h after the first dose of cloprostenol. Nellore and Simmental cows were artificially inseminated (12 and 24 h after LH injection) with frozen semen of proven fertility from the same Nellore and Simmental bulls used for IVF in the *in vitro* experimental groups. Embryo recovery (N = 80 and N = 74, respectively, for the Nellore and Simmental cows) was performed through uterine flushing with PBS on day 7.5 after the first AI, as previously described (Sudano, et al. 2011a). Recovered blastocysts were vitrified (N = 70) or subjected to the transcriptome analysis (a single blastocyst was used for each sample).

Vitrification

A two-step technique was used for blastocyst vitrification (N = 164), as previously described (Campos-Chillòn, et al. 2006). Briefly, blastocysts (grades 1 and 2) were washed in holding solution (HS; Vigro®, Bioniche Co.) and transferred to 500 μL of vitrification solution VS1 (5 M of ethylene glycol in HS) for 3 min. The embryos were then moved into 10 μL of vitrification solution VS2 (7 M ethylene glycol in HS + 0.5 M galactose + 18% (w/v) of Ficoll 70) for 45 s. A 0.25 mL straw containing 1 cm galactose solution (GS) (1 M of galactose in HS) + 0.5 cm air + 7 cm GS + 0.5 cm air + the drop of SV2 with the embryos + 0.5 cm air + GS (until the straw was full) was immediately mounted. Straws remained in liquid N_2 vapor for 1 min before being immersed in the liquid N_2.

Warming and subsequent culture of embryos

Vitrified straws were withdrawn from the liquid N_2 and kept in air at room temperature for 10 s, followed by 15 s in a 35–37°C water bath. The straws were slightly agitated to mix the columns of vitrification media. After 4 min, the embryos were washed and cultured in drops of SOFaa supplemented with 10% FCS (v/v), 2.7 mM myo-inositol, 0.2 mM pyruvate, 5 mg/mL BSA, 100 μg/mL streptomycin sulfate, and 100 iu/mL penicillin, which were covered with mineral oil. The embryos were kept in a humidified atmosphere of 5% CO_2 and 5% O_2 with the balance N_2 for 24 h to allow embryo re-expansion and hatching. Thereafter, the embryos were evaluated for their re-expansion (12 h under culture) and hatching/hatched (24 h under culture) rates. Embryo survival after vitrification/warming was defined as re-expansion followed by hatching of the blastocoele.

RNA extraction, amplification and microarray hybridization

Total RNA extraction from a single blastocyst per sample was performed using a PicoPure RNA isolation kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed by performing an on-column DNA digestion using RNase-free DNase (Qiagen, Mississauga, ON, Canada). RNA was eluted in 11 μl of elution buffer, and the quality of the extracted RNA was determined using a 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). Purified RNA was amplified by *in vitro* transcription with T7 RNA amplification in a two-round amplification protocol using a RiboAmp HS RNA amplification kit (Applied Biosystems). The concentration of

amplified RNA (aRNA) was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Biotin-labeled and fragmented cRNA were obtained using a 3'IVT Express kit (Affymetrix, Santa Clara, CA, USA) to perform hybridizations (N = 3–8) using a GeneChip Bovine Genome Array (Affymetrix) according to the manufacturer's instructions (Nellore IVP N= 4; Nellore ET N=4; Simmental IVP N=3; Simmental ET N=3). GeneChips were scanned using a GeneChip Scanner 3000 7G (Affymetrix), and the features extraction was performed using a Comand Console 3.2 (Affmetrix). Microarray data analysis was performed using FlexArray 1.6.1.1 (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007). FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL http://genomequebec.mcgill.ca/FlexArray). Data were subjected to a simple background correction, normalized within and between each array, and statistically analyzed through the use of linear models for microarray data analysis (LIMMA) (Smyth 2005). Genes with a fold-change of at least 1.5 and a probability of $P < 0.05$ were considered differentially expressed. Principal component analysis (PCA) was also performed using FlexArray 1.6.1.1 software.

Functional analysis, pathway analysis and network generation

Differentially expressed genes predominantly affected by subspecies, origin and interaction between both subspecies and origin were uploaded into the Ingenuity Pathways Analysis (IPA - Ingenuity Systems, www.ingenuity.com) and DAVID software (Huang, et al. 2009) to group overrepresented functions into clusters, to identify relationships between genes and to reveal common processes. The IPA functional analysis tool was used to identify the biological functions that were most significant in the data set. Canonical pathway analysis was also used to identify pathways from the IPA library that were most significant. For the network generation, a data set containing gene identifiers and corresponding expression values was uploaded into IPA. Networks were algorithmically generated based on information from the Ingenuity knowledge base. Genes are represented as nodes, and a biological relationship between two nodes is represented as edges (line).

Validation of microarray results using quantitative real-time PCR

The validation of microarray results was performed using quantitative real-time

PCR (qPCR). Amplified RNA from the samples submitted to microarray and independent additional samples (a single blastocysts per sample, totalizing n = 8 / group) were reverse transcribed using Oligo-dT primer and SuperScript III (Applied Biosystems), followed by qPCR, which was performed using TaqMan Universal Master Mix II (Applied Biosystems) and ABI 7500 (Applied Biosystems). As a control (to validate the RNA amplification), the extracted RNA from a pool of 200 IVP blastocysts was amplified or non-amplified and submitted to reverse transcription and qPCR of 17 genes (target and housekeeping genes described below) to perform Pearson's correlation analysis of mRNA abundances. Fourteen differentially expressed genes (Supplementary Table 1), regarding the main effects of subspecies, origin and interaction between subspecies and origin, were selected (target genes) for validation using qPCR, based on the functional and pathway analysis using IPA and DAVID software and the results of previous functional experiments from cryotolerance, lipid content and lipid composition (Sudano, et al. 2012b). The reference gene level was the geometric mean of the cycle threshold (Ct) values of *GAPDH*, *ACTB*, and *PPIA*. The relative expression of each gene was calculated using the $^{\Delta\Delta}$Ct method (Pfaffl 2001). Inventoried and made to order TaqMan gene expression assay primers were used to customize qPCR plates using the Custom Plate Service from Applied Biosystems (primer information is provided in Supplementary Table 1). Chilibot software was also used for network generation and to study the relationship between genes and related functions (Chen and Sharp 2004).

Statistical analysis

For *in vitro* and *in vivo* embryo production, the data were analyzed using the generalized linear mixed model (GLIMMIX) procedure with the SAS statistical software package (Version 9.2, SAS Inst. Inc., Cary, NC, USA). Sources of variation in the model included subspecies (*B. taurus indicus* - Nellore vs. *B. taurus taurus* - Simmental) and replicate were considered fixed and random effects, respectively. If necessary, the arcsine transformation was applied to percentage data to fit them into a normal distribution. The data are reported as untransformed least-squares means ± SEM.

Embryo re-expansion and hatching/hatched data were analyzed using the logistic regression (PROC LOGISTIC) in SAS. Sources of variation in the model included subspecies (*B. taurus indicus* - Nellore vs. *B. taurus taurus* - Simmental),

origin (IVP vs. ET), replicate and first order interactions. All factors, except replicate, were considered fixed effects. In the absence of significant interactions, only main effects are presented. Data are reported as percentages.

For the qPCR analysis, the data were analyzed with ANOVA using the generalized linear mixed model (GLIMMIX) procedure with SAS. Sources of variation in the model included subspecies (*B. taurus indicus* - Nellore vs. *B. taurus taurus* - Simmental), origin (IVP vs. ET), replicate and first-order interactions. All factors except replicate were considered fixed effects. If necessary, logarithmic transformation was applied to obtain a normal distribution of the data. In the absence of significant interactions, only main effects means are presented. The data are reported as untransformed least-squares means ± SEM. In addition, Pearson's correlation was calculated (PROC CORR in SAS) between amplified and non-amplified mRNA abundances. For all analyses, $P < 0.05$ was considered significant.

Results

Embryo production

There was no difference ($P > 0.05$) in the cleavage rates between the *in vitro*-produced Simmental and Nellore embryos (Table 1). However, the blastocyst per oocyte and blastocyst per cleaved embryo rates were higher ($P < 0.05$) in the Nellore embryos (Table 1). In the *in vivo* embryo production system, the total numbers of ova/embryos recovered and transferable embryos were not different ($P > 0.05$) between the Nellore and Simmental cows (Table 1).

Cryotolerance

The Simmental blastocysts had higher ($P < 0.05$) re-expansion and hatching/hatched rates than the Nellore embryos (Table 2), while the IVP embryos had lower ($P < 0.05$) postcryopreservation survival rates than the ET-produced embryos (Table 2).

Transcriptome analysis

A principal component analysis demonstrated that the four experimental groups could be resolved via their transcriptomic profiles. In the three-dimensional PCA-plot cluster, samples corresponding to different breeds (Nellore vs. Simmental) and origin

(IVP vs. ET) were clearly separated with a pronounced group individualization (Fig. 1).

Microarray analysis identified 158 differentially expressed genes ($P < 0.05$) between subspecies, with 152 and 6 genes up- and down-regulated, respectively, in the Simmental compared with the Nellore blastocysts (Supplementary Table 2 and Fig. 2). Moreover, 532 genes were differentially expressed ($P < 0.05$) between origin, with 516 and 16 genes down- and up-regulated, respectively, in the ET compared with the IVP embryos (Supplementary Table 3 and Fig. 2). With regards to the interaction effect between subspecies and origin, 53 genes were differentially expressed (Supplementary Table 4 and Fig. 2). A Venn diagram allows for a visualization of the distribution of the differentially expressed genes in relation to the main effects (subspecies and origin) and interaction between subspecies and origin (Fig. 2).

Functional classification and pathway analyses

The ontological classification of differentially expressed genes in the IPA software showed that nucleic acid metabolism (e.g., *ATP5B* and *ELOVL6*), cell-to-cell signaling and interaction (*GRP* and *CD46*), cell cycle (*CKS2* and *TPX2*), lipid metabolism (*AUH*, *ELOVL6* and *CYCS)*, carbohydrate metabolism (*UGP2* and *ALDOA*) and cell death and survival (*CD59* and *KRT19*) were important functional processes that differed ($P < 0.05$) between the Simmental and Nellore blastocysts (Fig. 3). Free radical scavenging (e.g., *CAT*, *GPX4*, *PRDX1* and *SOD2*), cell cycle (*CAT, SOD2* and *KRT18*), cell death and survival (*HSPA5, KRT18, CAT, GPX4*, *HSPA4*, *DNAJB1* and *DAP*), lipid metabolism (*ACSL3, ACSL6*, *CAT*, *GPX4* and *SCD*), cellular function and maintenance (*HSPA4*, *HSPA5, KRT18*, *CAT* and *GPX4*), and cellular development (*KRT19*, *KRT8, CAT*, *SOD1*, *SOD2* and *HSPA5*) were the major biological functions that differed ($P < 0.05$) between the IVP and ET blastocysts (Fig. 3). In addition, energy production (e.g., *CPT1A* and *FABP3*), lipid metabolism (*CPT1A*, *FABP3*, *PRDX2*, *CYP3A5* and *HSD17B10*), gene expression (*EIF2S3* and *EIF3G*), protein synthesis (*EIF2S3*, *EIF3G* and *RPS27L*), and amino acid (*ENPEP*) and carbohydrate metabolism (*CPT1A*, *FABP3* and *NUCB2*) were the significant biological functions/processes affected ($P < 0.05$) by the interaction between subspecies and origin (Fig. 3). The gene names are described in Supplementary Tables 2, 3 and 4. Several pathways were generated for each effect based on differentially expressed genes. The lipid metabolism and cell death and

survival shared pathways of the differentially expressed genes between IVP and ET generated by IPA are presented in Fig. 4.

Quantitative real-time PCR

To validate the RNA amplification, the amplified and non-amplified RNAs from a pool of 200 blastocysts were reversed transcribed and submitted to qPCR to quantify the mRNA abundances of 17 genes. The messenger RNA abundances of the amplified and non-amplified RNAs had a high correlation (r = 0.89, $P < 0.01$). Genes related to lipid metabolism (*ACSL3*, *ACSL6*, *AUH*, and *ELOVL6*), mitochondrial metabolism (*ATP5B*), oxidative stress (*CAT* and *GPX4*), heat shock (*HSPA5*), maternal recognition of pregnancy and pregnancy (*IFNT2* and *PAG2*), cell differentiation (*KRT18*) and apoptosis (*DAD1*, *DAP* and *PRDX2*) were selected (as previously described) for the validation of the microarray data (genes are listed in Supplementary Table 1). The qPCR validation confirmed differential expression levels for 85.7% of the selected genes (12/14). The expression levels of *AUH*, *ELOVL6*, *ATP5B* and *DAD1* were up-regulated ($P < 0.05$) in the Simmental compared with the Nellore blastocysts (Fig. 5), whereas *GPX4* was up-regulated ($P < 0.05$) in the Nellore blastocysts (Fig. 5). The expression levels of *ACSL3*, *ACSL6*, *HSPA5*, *IFNT2*, *KRT18* and *DAP* were up-regulated ($P < 0.05$) in the IVP compared with the ET blastocysts (Fig. 5). The interaction of subspecies and origin affected the mRNA abundance levels of *PAG2* and *PRDX2*, which were up-regulated ($P < 0.05$) in the Nellore IVP and Simmental ET blastocysts, respectively (Fig. 6). Moreover, the *PAG2* mRNA level was also up-regulated in the Nellore ET compared with the Simmental ET blastocysts. The relationship between validated genes and functional processes was obtained using a Chilibot interaction analysis, which is presented in Fig. 7.

Discussion

In the present study, we demonstrate that the cryotolerance and global gene expression patterns of *B. taurus indicus* (Nellore) and *B. taurus taurus* (Simmental) *in vitro*- and *in vivo*-produced blastocysts are different. In addition, we show that genes regulating lipid metabolism/composition and embryo viability are differentially expressed between groups.

Cryopreservation is a biotechnology that allows for the preservation of embryos for a prolonged period of time. Survival after cryopreservation is lower for embryos produced *in vitro* compared with those produced *in vivo* (Sudano, et al. 2011b), which has been attributed, at least in part, to the large amount of lipid droplets in the cytoplasm of IVP embryos (Abe, et al. 2002, Sudano, et al. 2011b). However, in addition to the amount of lipid droplets, lipid composition also affects embryo postcryopreservation survival (Sudano, et al. 2012b).

There are many differences in the reproductive physiology between *B. taurus indicus* and *B. taurus taurus*, including the number of oocytes recovered from OPU (Pontes, et al. 2011, Viana, et al. 2000) and their potential for *in vitro* embryo development (Pontes, et al. 2010). Indeed, more *in vitro*-produced blastocysts were achieved in the Nellore than in the Simmental cows (Table 1). However, this difference was not observed for ET embryos (Table 1), which is corroborated by a previous report (Wohlres-Viana, et al. 2011).

Information on differences between *B. taurus indicus* and *B. taurus taurus* embryos with regard to survival after cryopreservation and lipid content and/or composition is limited. Embryos produced *in vivo* from *B. taurus taurus* present a greater accumulation of cytoplasmic lipid droplets than those from *B. taurus indicus*, despite the fact that *B. taurus taurus* embryos have a higher cryotolerance (Visintin, et al. 2002). Arreseigor et al. (Arreseigor 1998) reported higher pregnancy rates for frozen *B. taurus taurus* ET embryos compared with those of *B. taurus indicus*. Previous results from our laboratory also indicate a higher lipid content in Simmental embryos compared with Nellore, which points to differences in the membrane phospholipid composition between these breeds and between embryos produced *in vitro* or *in vivo* (Sudano, et al. 2012b). These observations are likely related to a higher embryo cryotolerance for Simmental and ET embryos in comparison with Nellore and IVP embryos, as observed in the present study (Table 2).

A variety of genes was recently found to be differentially expressed in fresh or vitrified embryos produced *in vivo* vs. *in vitro* (Aksu, et al. 2012). Differences in the global gene expression patterns between IVP and ET and also between Nellore and Simmental were addressed by PCA analysis in the present study (Fig. 1), and a large number of differentially expressed genes between subspecies and origin were identified by transcriptome analysis (Fig. 2, and Supplementary Tables 2, 3 and 4). Functional and pathway analyses revealed that several ontological processes were

associated with differentially expressed genes (Figs. 3 and 4). Pathways controlling free radical scavenging, cell death and survival, cell cycle and lipid and carbohydrate metabolism could be promptly identified and were compatible with previous data on lipid content and composition (Sudano, et al. 2012b) and also could be related with postcryopreservation survival (Table 2).

Gene overexpression is characteristic of IVP compared with ET bovine embryos (Corcoran, et al. 2006, Côté, et al. 2011). This result was observed in the present study (Supplementary Table 2, Figs. 2 and 5B) and might be explained by an active unquiet metabolism (Côté, et al. 2011, Leese 2002). Leese et al. (Leese, et al. 2008) described that the metabolism of IVP embryos, which is defined as a "loss of quietness", is up-regulated and determined by exposure to environmental stress, leading to the generation of reactive oxygen species (ROS) and oxidative stress. Indeed, free radical scavenging was the major biological function affected by the origin effect (Fig. 3), and the mRNA abundance levels of the genes *HSPA5*, *IFNT2*, *KRT18* and *DAP* were also up-regulated in the IVP embryos (Fig. 5B), which could be associated with a reduced embryo viability followed by a reduction of the survival after cryopreservation.

The expression of heat shock protein family genes is already known to be related to a stress response under unfavorable embryo conditions, including heat shock (Hansen 2009, Paula-Lopes and Hansen 2002) and the temporal sequence of cold-induced expression after a return to normal temperatures (Sonna, et al. 2002). The gene *IFNT2* is frequently correlated with the maternal recognition of pregnancy and embryo viability (Rizos, et al. 2003); however, a higher abundance of mRNA encoding this gene is related to embryonic stress response under high oxygen tension (Rodina, et al. 2009) and after cryopreservation (Stinshoff, et al. 2011). *DAP* is an apoptosis-related gene, and its up-regulation is described in "DDK syndrome", in which embryonic death occurs at the morula-blastocyst transition in mice (Hao, et al. 2009). Another gene up-regulated in IVP blastocysts is *KRT18*, an embryonic trophectoderm cell differentiation marker (Ozawa, et al. 2012) associated with intermediate filaments of the cytoskeleton and blastocyst formation (Marceau, et al. 2004). However, this gene appears to have a key function in the caspase regulation activity of cells responding to apoptotic stimuli (Marceau, et al. 2004) and could be connected with the overexpression of *IFNT2*. All of these genes could be associated with our findings (Fig. 5B and Table 2).

Lipid metabolism in embryonic cells is still not completely understood (McKeegan and Sturmey 2011). The long chain acyl-CoA synthetases (ACSLs – isoforms 1 to 6) are an integral initial step for virtually every metabolic pathway that degrades fatty acids and synthesizes complex lipids, including β-oxidation, desaturation and elongation reactions (Coleman, et al. 2002, de Jong, et al. 2007). In addition, the involvement of ACSLs in fatty acid uptake has been demonstrated in different types of cells (Dutta-Roy 2000, Johnsen, et al. 2009). The observed up-regulation of the genes *ACSL3* and *ACSL6* in IVP embryos (Fig. 5B) could likely be linked with an uptake of lipids from the culture medium, resulting in higher amounts of lipids present in their cytoplasm and more saturated phospholipid species observed in the cellular membranes of these embryos (Sudano, et al. 2011b, Sudano, et al. 2012b). In contrast, the lipid metabolism-related genes *AUH* and *ELOVL6*, which are more precisely associated with an enoyl-CoA hydratase activity of β-oxidation (Nakagawa, et al. 1995) and the elongation of unsaturated fatty acids (Jump 2009, Moon, et al. 2009), respectively, were up-regulated in the Simmental blastocysts (Fig. 5A), which could be associated with an increased metabolization of fatty acids that culminates with more unsaturated phospholipids observed in the cellular membranes of these embryos (Sudano, et al. 2012b). Moreover, *ATP5B*, which is related to proton transport and ATP generation during oxidative phosphorylation, was also up-regulated in the Simmental blastocysts (Fig. 5A), which is in agreement with the idea of an increased metabolization activity of these embryos.

Furthermore, the Simmental blastocysts presented an increased *DAD1* mRNA level (Fig. 5A), a gene that prevents apoptosis and cell death (Brewster, et al. 2000, Nishii, et al. 1999). Deletion of *DAD1* in mice induces apoptosis-associated embryonic death (Brewster, et al. 2000), and its transgenic expression in *Caenorhabditis elegans* using a heat shock promoter revealed that heat-shocked transgenic animals exhibit a 40% decrease in programmed cell death during early embryonic development (Sugimoto, et al. 1995). On the other hand, the Nellore embryos displayed higher levels of *GPX4* (Fig. 5A), indicating possible increased oxidative stress (Ufer and Wang 2011). These results could explain the higher susceptibly of Nellore blastocysts to cryopreservation in comparison with Simmental blastocysts (Table 2).

PAG2 and *PRDX2* were affected by an interaction between subspecies and origin (Fig. 6). *PAG2* is a trophoblastic-specific transcript member of the large

pregnancy-associated glycoprotein gene family (Szafranska, et al. 2001). Several studies have described the use of pregnancy-associated glycoprotein blood plasma concentrations for early pregnancy diagnoses (Humblot, et al. 1988, Sousa, et al. 2006). The expression of *PAG2* genes in extraembryonic membranes, endometrial tissue and day 17 conceptus have already been described (Thompson, et al. 2012). There is currently no report in the literature on *PAG2* expression in early developmental stage embryos. In the present trial, the *PAG2* mRNA levels were higher in the Nellore IVP embryos than in the other groups, which was also true for the Nellore ET compared with the Simmental ET (Fig. 6). Higher levels of pregnancy-associated glycoprotein genes have also been observed in extraembryonic membranes, caruncular tissue and the plasma of pregnant cows bearing cloned embryos (Chavatte-Palmer, et al. 2006, Constant, et al. 2011). However, the functional role of the *PAG2* gene in blastocysts remains undetermined. The *PRDX2* gene is a member of an antioxidant enzyme family that modulates H_2O_2 production and H_2O_2-mediated apoptosis (Kim, et al. 2000). Higher levels of *PRDX2* expression have been associated with a greater viability of oocytes and embryos (Lee, et al. 2010, Leyens, et al. 2004). However, in the present study, the higher *PRDX2* level observed in the Simmental ET embryos still required to be elucidated once no difference was observed with Nellore IVP blastocysts (Fig. 6).

The Simmental and ET blastocysts had higher post-cryopreservation survival rates than the Nellore and IVP embryos, respectively. Differences of gene expression patterns involved in the free radical scavenging, lipid and acid nucleic metabolism, cell death and survival were identified. The genes *AUH* and *ELOVL6*, *ACSL3* and *ACSL6*, which were up-regulated in Simmental and IVP blastocysts, respectively, could explain the different lipid compositions and postcryopreservation survival capacities (Fig. 7). Genes related to mitochondrial metabolism (*ATP5B*), oxidative stress (*GPX4*), apoptosis (*DAD1*, *DAP*, *PRDX2*), heat shock (*HSPA5*), pregnancy (*IFNT2*, *PAG2*), and cell differentiation (*KRT18*) could elucidate the different levels of embryo viability and cryotolerance (Fig. 7). In conclusion, the results generated in this study provide different transcriptomic signatures of a model with two bovine subspecies and two embryo origins that presented a characteristic lipid content, lipid profile and postcryopreservation survival.

Declaration of interest

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

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Table 1

In vitro (IVP) and in vivo (ET) production of embryos from Nellore and Simmental cows (least-squares means ± SEM, when applicable).

Origin	Parameter	Subspecies		*P-value*
		Bos taurus indicus (Nellore)	*Bos taurus taurus* (Simmental)	
IVP	Cows (no.)	14	14	-
	Oocytes (no.)	840	450	-
	Cleavage (%)	87.1 ± 1.0	86.4 ± 1.0	0.57
	Blastocyst / oocyte (%)	41.5 ± 1.9[a]	23.4 ± 1.9[b]	< 0.01
	Blastocyst / cleaved (%)	47.7 ± 2.0[a]	27.0 ± 2.0[b]	< 0.01
ET	Cows (no.)	7	8	-
	Ova / embryos recovered (no.)*	5.5 ± 0.9	3.7 ± 0.8	0.14
	Transferable embryos (no.)*	3.8 ± 1.0	2.3 ± 0.8	0.23

[a,b] Within a row, values without a common superscript differ (P < 0.05).

* Least-squares means per cow per session.

Table 2
Re-expansion (12 h) and hatching / hatched (24 h) rates of *Bos taurus indicus* and *Bos taurus taurus* in vitro- (IVP) and in vivo- (ET) produced blastocysts after cryopreservation, warming, and re-culture.

Parameter	Subspecies			Origin		
	Bos taurus indicus (Nellore)	*Bos taurus taurus* (Simmental)	*P* value	IVP	ET	*P* value
Re-expansion % (no.)	69.6 (62 / 89)[a]	86.6 (65 / 75)[b]	0.02	68.1 (64 / 94)[A]	90.0 (63 / 70)[B]	< 0.01
Hatching / hatched % (no.)	20.2 (18 / 89)[a]	34.6 (26 / 75)[b]	0.04	18.1 (17 / 94)[A]	38.5 (27 / 70)[B]	< 0.01

[a,b] Within a row, percentage without a common superscript differ ($P < 0.05$).

[A,B] Within a row, percentage without a common superscript differ ($P < 0.05$).

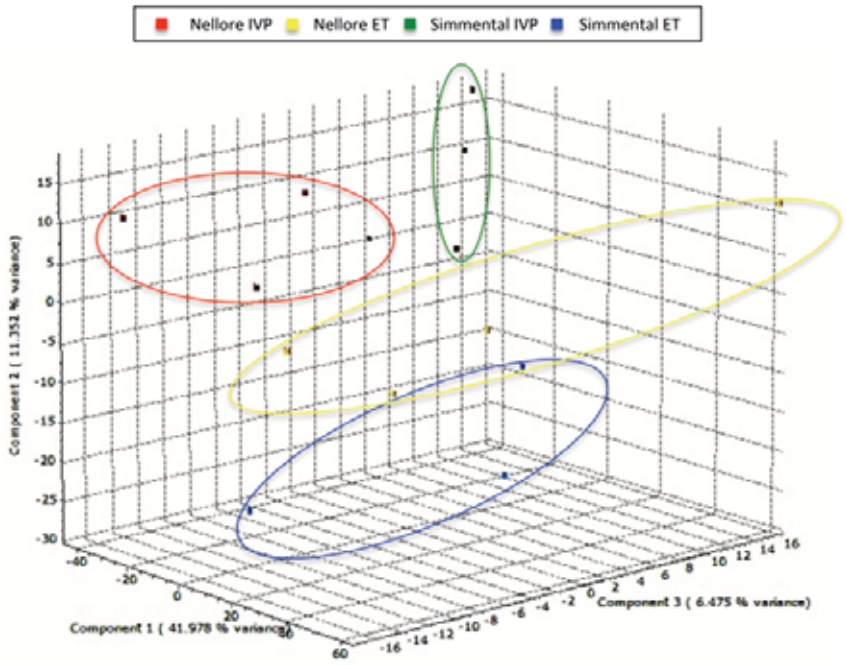

Figure 1

Three-dimensional PCA plot for the microarray data for the Nellore and Simmental IVP and ET blastocysts (n = 3–4 per group).

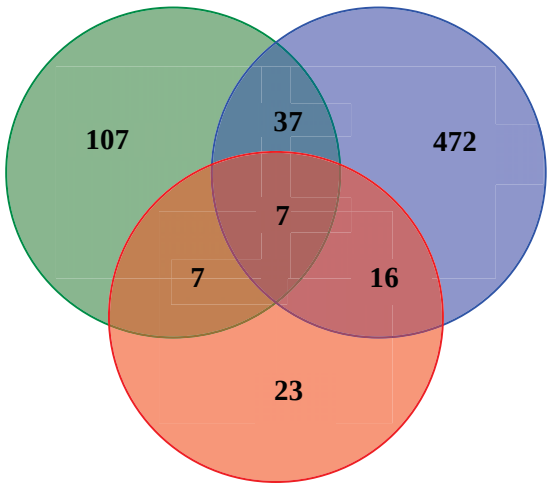

Figure 2

Venn diagram of genes differentially expressed between subspecies (green – 158 genes, with 152 and 6 genes up- and down-regulated, respectively, in Simmental compared with Nellore blastocysts), origin (blue – 532 genes, with 516 and 16 genes down- and up-regulated in ET- compared with IVP-derived blastocysts, respectively) and interactions between subspecies and origin (red – 53 genes). Intersections indicate common genes.

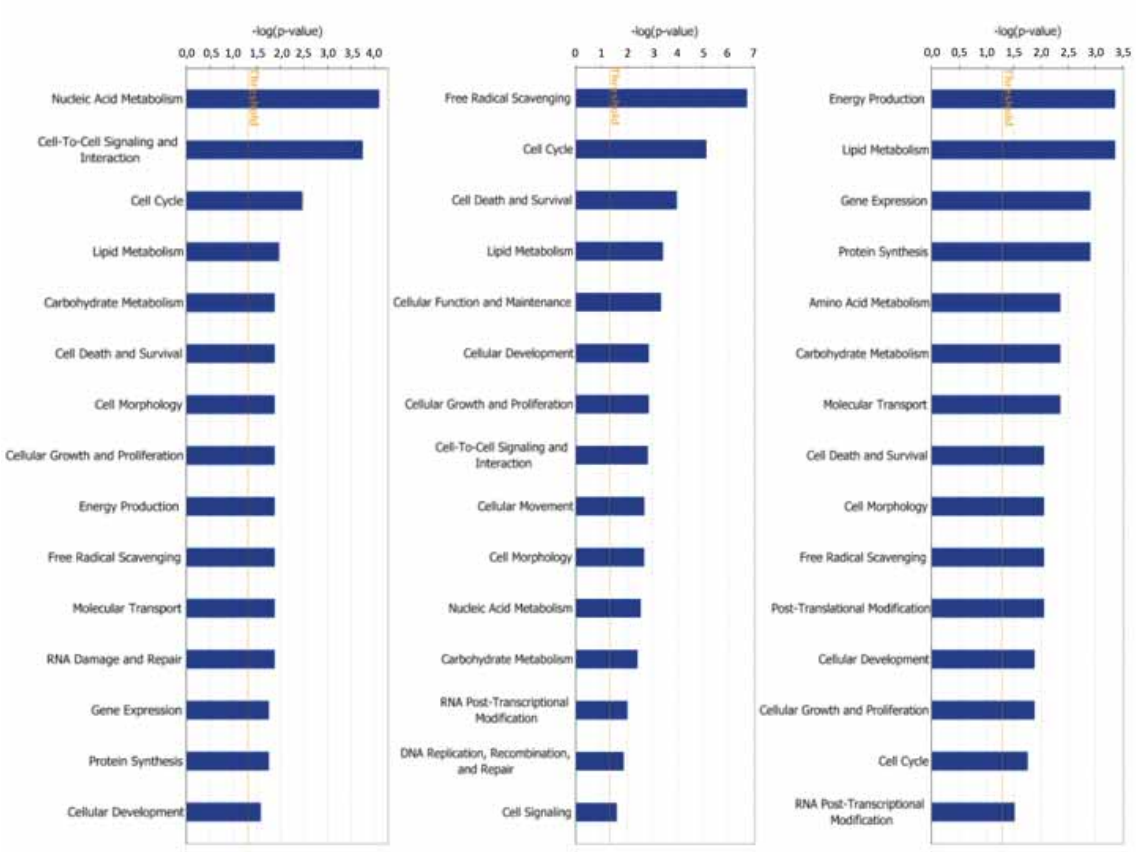

Figure 3

Functional grouping of differentially expressed genes with regards to the main effects of subspecies, origin and the interaction between subspecies and origin (left to right, respectively) using Ingenuity Pathways Analysis software. The main significant functional groups ($P < 0.05$) are presented graphically. The bars represent the *P*-value on a logarithmic scale for each functional group.

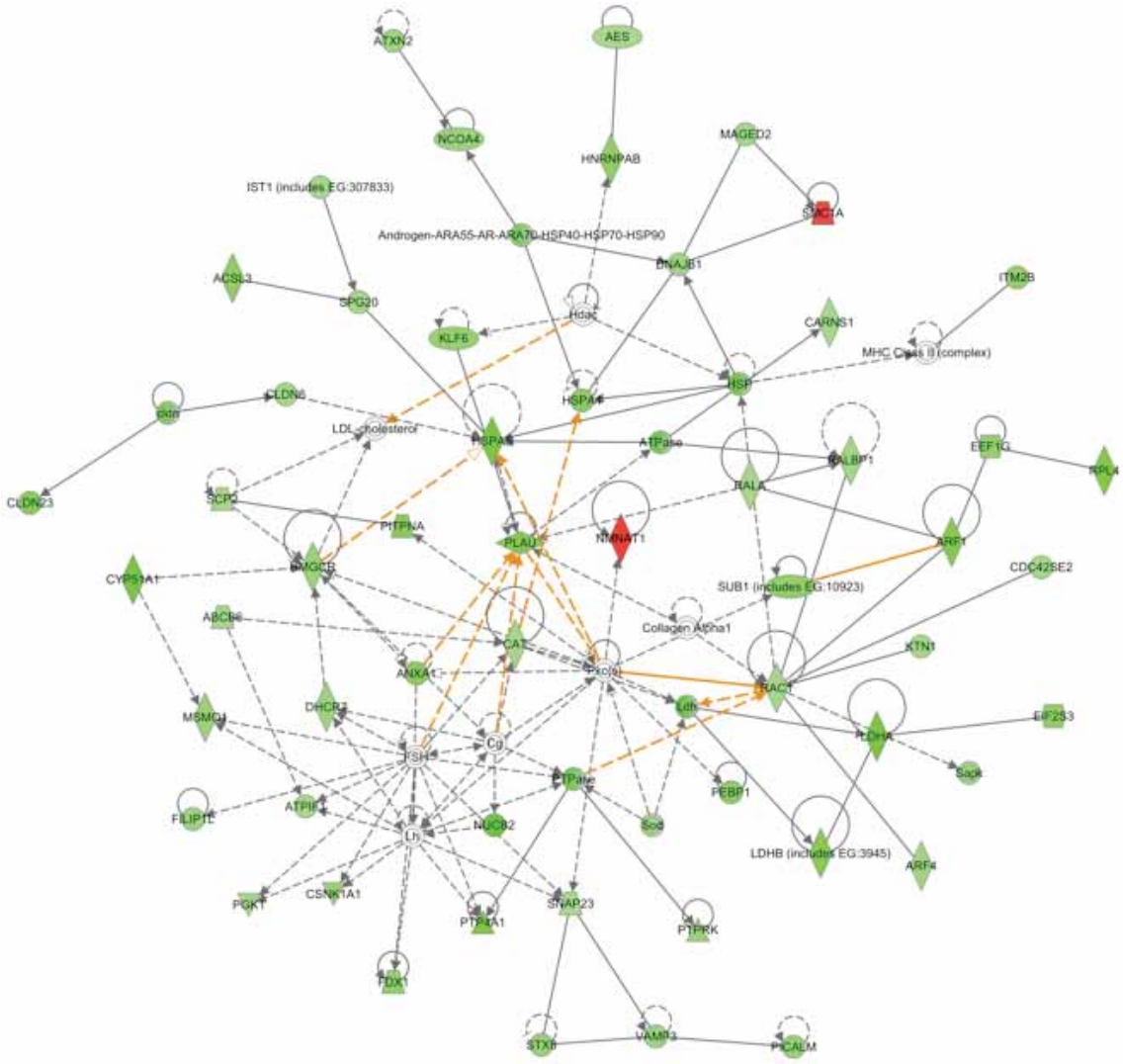

Figure 4

Ingenuity pathway interaction network analysis. Genes involved in lipid metabolism and the cell death and survival shared pathway, which were differentially expressed between the IVP- and ET-produced blastocysts, are presented. The network displays nodes (genes/gene products) and edges (the biological relationship between nodes). Gene node color intensity indicates increased (red) or decreased (green) fold changes in the ET compared with the IVP blastocysts. Relationships between molecules are indicated by solid (direct) or dashed (indirect) connecting lines, and the yellow color indicates shared nodes among the pathways.

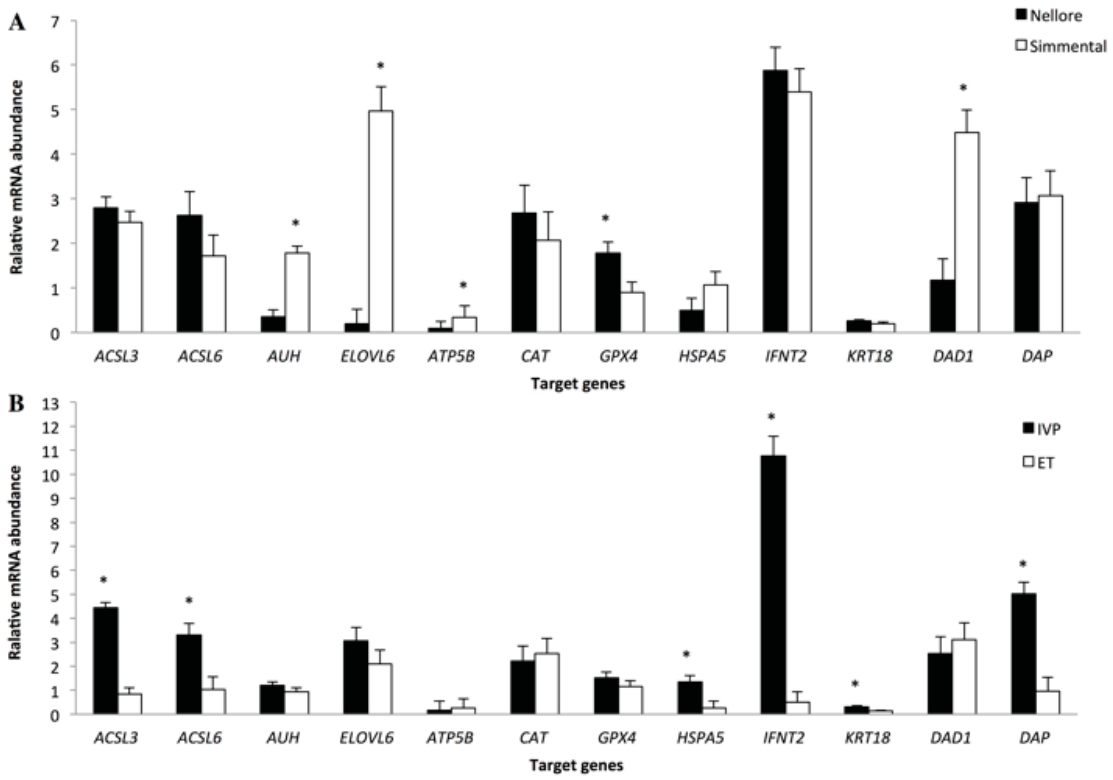

Figure 5.

Subspecies (A) and origin (B) effect in the mRNA abundance of differentially expressed genes in the Nellore and Simmental in vitro- (IVP) and in vivo- (ET) produced blastocysts (least-squares means ± SEM). *Different ($P < 0.05$). N = 16 per group. Gene names are described in Supplemental Table S1.

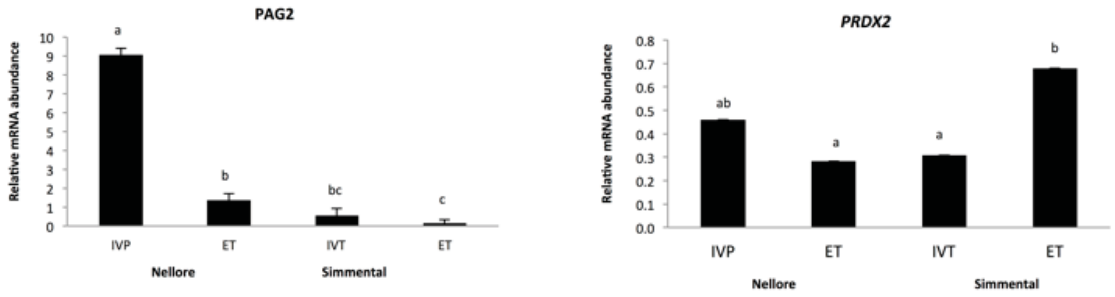

Figure 6.

Relative mRNA abundance of genes with interaction effect between subspecies and origin (*PAG2 e PRDX2*) from Nellore and Simmental in vitro- (IVP) and in vivo- (ET) produced blastocysts (least-squares means ± SEM). [abc]Uncommon superscripts differ ($P < 0.05$). N = 8 per group. Gene names are described in Supplemental Table S1.

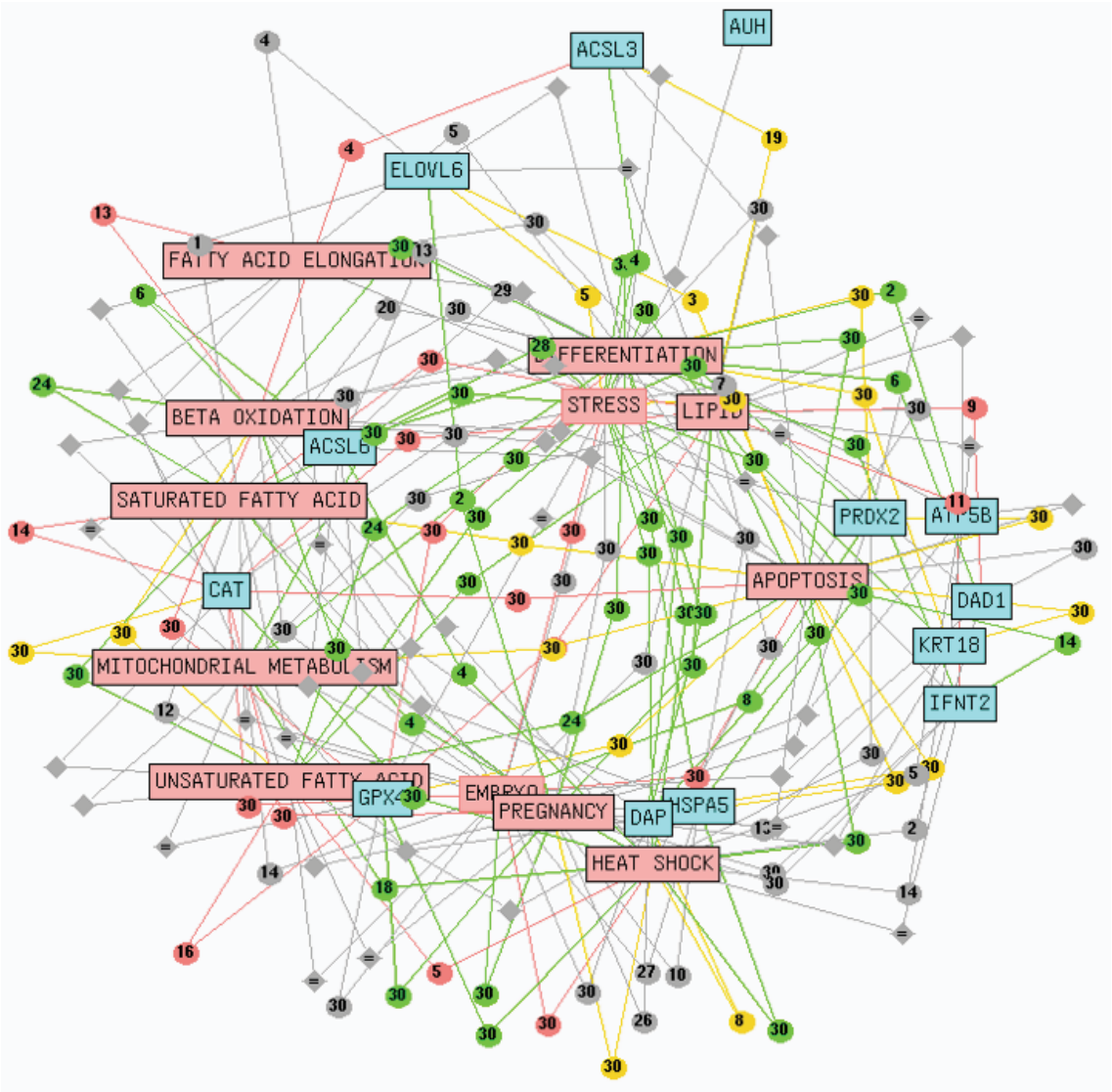

Figure 7

Relationship between validated genes and associated functional processes obtained through a Chilibot interaction analysis. Lines connecting rectangular nodes indicate relationships between the genes and related functions. Each icon in the middle of a line represents the character of the relationship. Interactive relationships (circles) are neutral (gray), stimulatory (green), inhibitory (red) or both stimulatory/inhibitory (yellow). The number within each icon indicates the quantity of abstracts retrieved for documenting that relationship. Icons containing the equal sign ("=") represent "parallel relationships". Gray rhomboidal icons indicate that only a co-occurrence was detected.

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Supplementary Table 1.
Information of reverse transcription qPCR-specific primers of target and housekeeping genes expressed in bovine blastocysts.

Gene symbol	**Gene Name**	**Accession number**	**Taqman Assay***
ACSL3	acyl-CoA synthetase long-chain family member 3	NM_001205468.1	Bt04282139_m1
ACSL6	acyl-CoA synthetase long-chain family member 6	BC111155.1	Bt03231695_m1
AUH	AU RNA binding protein/enoyl-CoA hydratase	NM_001105436.1	Bt03275798_m1
ELOVL6	ELOVL fatty acid elongase 6	NM_001102155.1	Bt00907566_m1
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	NM_175796.2	Bt03216728_m1
CAT	catalase	BC103066.1	Bt03228716_m1
GPX4	glutathione peroxidase 4	NM_174770.3	Bt03259613_g1
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	NM_001075148.1	Bt03244880_m1
IFNT2	interferon tau	NM_001015511.3	Bt03210579_g1
PAG2	pregnancy-associated glycoprotein 2	NM_176614.1	Bt03292796_gH
KRT18	keratin 18	NM_001192095.1	Bt00989202_g1
DAD1	defender against cell death 1	NM_001034761.1	Bt03221619_m1
DAP	death-associated protein	BC103332.1	Bt03230650_m1
PRDX2	peroxiredoxin 2	NM_174763.2	Bt03216179_g1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	AB098979.1	Bt03210913_g1
ACTB	actin, beta	NM_173979.3	Bt03279174_g1
PPIA	peptidylprolyl isomerase A (cyclophilin A)	NM_178320.2	Bt03224614_g1

* Applied Biosystems Taqman Gene Expression Assays Reference.

Supplementary Table 2.
List of differentially expressed transcripts between Simmental and Nellore blastocysts.

Representative Public ID	Gene Title	Gene Symbol	Fold change	*P*-value
NM_174133.2	polyubiquitin	*LOC281370*	3.42	0.0107
CK980192	4112256 BARC 9BOV Bos taurus cDNA clone 9BOV41_N23 3-, mRNA sequence	---	2.81	0.0411
BP107621	gastrin-releasing peptide	*GRP*	2.80	0.0131
BE749511	tripartite motif-containing 2	*TRIM2*	2.79	0.0011
NM_174133.2	polyubiquitin	*LOC281370*	2.66	0.0142
CK943278	F11 receptor	*F11R*	2.60	0.0326
CB425639	placenta-specific 8	*PLAC8*	2.59	0.0059
AU278102	CDGSH iron sulfur domain 1	*CISD1*	2.59	0.0331
CK849176	glutamic pyruvate transaminase (alanine aminotransferase) 2	*GPT2*	2.56	0.0116
CK953255	aldehyde dehydrogenase 2 family (mitochondrial)	*ALDH2*	2.55	0.0032
CB420281	desmoglein 2	*DSG2*	2.51	0.0394
CK978263	RAB10, member RAS oncogene family	*RAB10*	2.45	0.0251
CK952849	similar to ribosomal protein S4, X-linked X /// similar to ribosomal protein S4, X-linked X /// similar to ribosomal protein S4, X-linked X /// ribosomal protein S4, X-linked /// ribosomal protein S4, Y-linked 1 /// ribosomal protein S4, Y-linked 2	*LOC781612 /// LOC783463 /// LOC784060 /// RPS4X /// RPS4Y1 /// RPS4Y2*	2.40	0.0235
AY528254.1	COX5A protein	*COX5A*	2.34	0.0307
CK777089	mitochondrial ribosomal protein L22	*MRPL22*	2.33	0.0096
CK959803	chaperonin containing TCP1, subunit 6A (zeta 1)	*CCT6A*	2.29	0.0001
CK848474	myoferlin	*MYOF*	2.29	0.0017
AY342429.1	CD46 molecule, complement regulatory protein	*CD46*	2.20	0.0249
M62428.1	ubiquitin C	*UBC*	2.20	0.0122
CB424390	testis expressed 12	*TEX12*	2.17	0.0055

CB461078	721004 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	2.15	0.0047
CK948740	pyruvate dehydrogenase (lipoamide) alpha 1	*PDHA1*	2.15	0.0202
BP110236	CDC28 protein kinase regulatory subunit 2	*CKS2*	2.13	0.0296
AV618456	pallidin homolog (mouse)	*PLDN*	2.12	0.0003
BI535743	glycogen synthase kinase 3 alpha	*GSK3A*	2.11	0.0298
NM_174025.2	crystallin, zeta (quinone reductase)	*CRYZ*	2.10	0.0104
BP108263	secreted seminal-vesicle Ly-6 protein 1	*SSLP1*	2.09	0.0065
CB533459	succinate-CoA ligase, ADP-forming, beta subunit	*SUCLA2*	2.09	0.0188
CK966474	neuroepithelial cell transforming gene 1	*NET1*	2.09	0.0203
BP105001	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	*PIP4K2A*	2.08	0.0092
NM_174760.2	ribosomal protein L10	*RPL10*	2.06	0.0447
AW656417	eukaryotic translation termination factor 1	*ETF1*	2.05	0.0444
CK848597	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	*ATP5C1*	2.05	0.0145
AV601802	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	*ATP5A1*	2.05	0.0487
CB419743	Transmembrane protein 127	*TMEM127*	2.03	0.0006
CB535261	768672 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	2.03	0.0129
BP102330	ribosomal protein L39	*RPL39*	2.02	0.0325
BM435101	zinc finger protein 36, C3H type-like 1	*ZFP36L1*	2.02	0.0057
NM_174778.1	ribosomal protein S27a	*RPS27A*	1.99	0.0494
AV615649	tetraspanin 6	*TSPAN6*	1.98	0.0297
AV598939	reticulon 3	*RTN3*	1.98	0.0003
NM_175796.2	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	*ATP5B*	1.97	0.0327
CK961717	asparaginyl-tRNA synthetase	*NARS*	1.97	0.0242
CB451598	chaperonin containing TCP1, subunit 4 (delta)	*CCT4*	1.97	0.0368
CB166358	lysosomal-associated membrane protein 2	*LAMP2*	1.97	0.0094

CK848991	prosaposin	*PSAP*	1.95	0.0322
CK959800	synaptosomal-associated protein, 23kDa	*SNAP23*	1.94	0.0043
CB463704	wntless homolog (Drosophila)	*WLS*	1.94	0.0036
NM_174099.2	lactate dehydrogenase A	*LDHA*	1.93	0.0193
CB170586	tumor-associated calcium signal transducer 2	*TACSTD2*	1.92	0.0202
CB424217	COX4 neighbor	*COX4NB*	1.89	0.0225
BE751816	optineurin	*OPTN*	1.88	0.0196
BM435937	polymerase (DNA-directed), epsilon 4 (p12 subunit)	*POLE4*	1.88	0.0122
CK772572	glycerophosphodiester phosphodiesterase 1	*GDE1*	1.87	0.0122
NM_174334.2	hydroxysteroid (17-beta) dehydrogenase 10	*HSD17B10*	1.86	0.0187
AW313986	phosphatidylinositol glycan anchor biosynthesis, class B	*PIGB*	1.84	0.0016
AU232438	heat shock 27kDa protein 1	*HSPB1*	1.84	0.0144
BP107757	ribosomal protein S20	*RPS20*	1.81	0.0103
CB172313	ribosomal protein L7-like 1	*RPL7L1*	1.81	0.0018
CB426729	RNA pseudouridylate synthase domain containing 3	*RPUSD3*	1.81	0.0227
NM_175795.2	cytochrome c oxidase subunit VIIb	*COX7B*	1.81	0.0072
CB533274	tropomyosin 3	*TPM3*	1.80	0.0389
CB433758	non-POU domain containing, octamer-binding	*NONO*	1.79	0.0428
BE681641	tetratricopeptide repeat domain 9C	*TTC9C*	1.79	0.0025
CK775657	LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	*LSM7*	1.78	0.0405
CK770115	translocase of inner mitochondrial membrane 8 homolog A (yeast)	*TIMM8A*	1.78	0.0023
BM431714	1Duo26D1.ab1 Bos taurus Duodenum #1 library Bos taurus cDNA, mRNA sequence	---	1.78	0.0002
CB531801	signal sequence receptor, delta (translocon-associated protein delta)	*SSR4*	1.77	0.0005
CK960423	cytochrome c, somatic /// similar to cytochrome c-like protein	*CYCS /// LOC100138364*	1.76	0.0310
CK955868	arginine and glutamate rich 1	*ARGLU1*	1.76	0.0234

BE758011	zinc and ring finger 1	*ZNRF1*	1.76	0.0078
CK955677	keratin 19	*KRT19*	1.75	0.0294
BE721168	ubiquitin-conjugating enzyme E2F (putative)	*UBE2F*	1.75	0.0277
CK975252	ubiquinol-cytochrome c reductase complex 7.2 kDa protein	*UQCR10*	1.73	0.0417
CB443641	valosin containing protein (p97)/p47 complex interacting protein 1	*VCPIP1*	1.73	0.0216
CB460291	sorting nexin 6	*SNX6*	1.73	0.0201
CK846588	ribosomal protein L26-like 1	*RPL26L1*	1.73	0.0152
CK848926	protein disulfide isomerase family A, member 6	*PDIA6*	1.72	0.0139
CK772515	claudin 6	*CLDN6*	1.72	0.0214
CK972168	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	*ELOVL6*	1.71	0.0180
CB533579	RAB10, member RAS oncogene family	*RAB10*	1.71	0.0479
BP107394	ribosomal protein L21	*RPL21*	1.71	0.0381
CK848419	cell division cycle associated 8	*CDCA8*	1.71	0.0119
CK773855	eukaryotic translation elongation factor 1 gamma	*EEF1G*	1.71	0.0245
CB170168	SAR1 homolog A (S. cerevisiae)	---	1.70	0.0157
CK771620	similar to G protein-coupled receptor	*LOC782069*	1.70	0.0075
CK772180	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	*ATP6V0D2*	1.70	0.0476
CK778641	mutS homolog 6 (E. coli)	*MSH6*	1.70	0.0233
CK775344	mitochondrial ribosomal protein S15	*MRPS15*	1.69	0.0255
CB421885	proteasome (prosome, macropain) subunit, beta type, 2	*PSMB2*	1.69	0.0407
CB441821	similar to mCG142710	*LOC511229*	1.69	0.0090
CK944937	cytoskeleton associated protein 2	*CKAP2*	1.68	0.0404
CB168209	Influenza virus NS1A binding protein	*IVNS1ABP*	1.67	0.0118
BM256963	DEAD (Asp-Glu-Ala-As) box polypeptide 19A	*DDX19A*	1.67	0.0413
CB425905	basic leucine zipper and W2 domains 1	*BZW1*	1.66	0.0187
BM434841	PREDICTED: PWWP domain-containing protein 2A	*PWWP2A*	1.66	0.0389
BP109396	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	*SLC25A20*	1.66	0.0104

BP102572	CD59 molecule, complement regulatory protein	*CD59*	1.65	0.0490
CK975531	biliverdin reductase A	*BLVRA*	1.65	0.0071
CK971899	CDC5 cell division cycle 5-like (S. pombe)	*CDC5L*	1.65	0.0258
CK848123	ribosomal protein S24; similar to ribosomal protein S24	---	1.64	0.0073
BE682761	181057 MARC 4BOV Bos taurus cDNA 5-, mRNA sequence	---	1.64	0.0027
AU278120	transmembrane protein 209	*TMEM209*	1.63	0.0038
CB433125	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	*DDX3X*	1.63	0.0077
CK951444	transmembrane protein 20	*TMEM20*	1.63	0.0137
CK959397	polymerase (RNA) II (DNA directed) polypeptide F	*POLR2F*	1.62	0.0471
CK772522	karyopherin alpha 3 (importin alpha 4)	*KPNA3*	1.62	0.0076
NM_174212.1	UDP-glucose pyrophosphorylase 2	*UGP2*	1.62	0.0286
CK769700	abhydrolase domain containing 11	*ABHD11*	1.61	0.0081
CK948835	sorbin and SH3 domain-containing protein 1	*SORBS1*	1.61	0.0083
CK950633	kinesin family member 23	*KIF23*	1.61	0.0125
CB461980	heme binding protein 1	*HEBP1*	1.61	0.0009
CB451616	706397 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	1.61	0.0008
CK772462	mitochondrial ribosomal protein L39	*MRPL39*	1.61	0.0027
CK777982	cystatin B (stefin B)	*CSTB*	1.60	0.0426
CB428736	BCL2-associated athanogene 2	*BAG2*	1.60	0.0363
BE236706	galactose mutarotase (aldose 1-epimerase)	*GALM*	1.60	0.0073
NM_175803.2	tubulin folding cofactor A	*TBCA*	1.60	0.0334
CB168605	similar to ribophorin I	*LOC617690*	1.60	0.0325
CB166510	splicing factor 3b, subunit 2, 145kDa	*SF3B2*	1.59	0.0254
BE665193	rabaptin, RAB GTPase binding effector protein 1	*RABEP1*	1.59	0.0193
CB467596	transmembrane protein 167A	*TMEM167A*	1.59	0.0003
NM_174465.2	SEC11 homolog A (S. cerevisiae)	*SEC11A*	1.59	0.0441
AV602127	eukaryotic translation elongation factor 1 alpha 1	*EEF1A1*	1.59	0.0044
NM_175822.2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4,	*NDUFB4*	1.59	0.0060

	15kDa			
CK947713	transmembrane protein 49	*TMEM49*	1.58	0.0361
AV607802	nuclear receptor coactivator 1	*NCOA1*	1.57	0.0032
CB451234	ribosomal protein L36a-like	*RPL36AL*	1.57	0.0164
CK775424	ecdysoneless homolog (Drosophila)	*ECD*	1.57	0.0116
CK775235	polymerase (RNA) I polypeptide E, 53kDa	*POLR1E*	1.57	0.0039
CK967668	calpain 2, (m/II) large subunit	*CAPN2*	1.57	0.0180
CK965677	aldolase A, fructose-bisphosphate	*ALDOA*	1.56	0.0058
CK770196	mitochondrial ribosomal protein L16	*MRPL16*	1.56	0.0174
CB461416	TBC1 domain family, member 4	*TBC1D4*	1.55	0.0081
BP109944	solute carrier family 30 (zinc transporter), member 5	*SLC30A5*	1.55	0.0068
CK772666	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	*BUB1B*	1.54	0.0059
CK845911	phospholipase C, gamma 1	---	1.54	0.0469
NM_176613.2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	*ATP5G2*	1.54	0.0370
BM087625	reticulon 4 interacting protein 1	*RTN4IP1*	1.53	0.0088
CB533320	defender against cell death 1	*DAD1*	1.53	0.0355
CK776397	overexpressed breast tumor protein homolog	*TOMM6*	1.52	0.0334
CK771323	chromosome 19 open reading frame 63 ortholog	*C18H19ORF63*	1.52	0.0260
BG937530	TWIST neighbor	*TWISTNB*	1.52	0.0002
CK849084	chromosome 14 open reading frame 147 ortholog	*C21H14orf147*	1.52	0.0061
CK778368	TPX2, microtubule-associated, homolog (Xenopus laevis)	*TPX2*	1.51	0.0206
BF046219	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa	*SNRPD2*	1.51	0.0223
CK967463	vacuolar protein sorting 29 homolog (S. cerevisiae)	*VPS29*	1.51	0.0216
BP102272	X (inactive)-specific transcript	*XIST*	1.51	0.0482
CK849726	coiled-coil-helix-coiled-coil-helix domain containing 3	*CHCHD3*	1.50	0.0269
CK965203	AU RNA binding protein/enoyl-CoA hydratase	*AUH*	1.50	0.0145
CK775497	fibrillarin	*FBL*	1.50	0.0251
BE237565	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	*SUGT1*	1.50	0.0262

CK953132	aldo-keto reductase family 1, member A1 (aldehyde reductase)	*AKR1A1*	-1.61	0.0053
BF605641	zinc ribbon domain containing 1	*ZNRD1*	-1.71	0.0062
CB426568	synaptonemal complex protein 3	*SYCP3*	-1.98	0.0190
BP108137	BP108137 ORCS bovine utero-placenta cDNA Bos taurus cDNA clone ORCS116263-, mRNA sequence	---	-1.99	0.0004
CB440375	interleukin 33	*IL33*	-2.01	0.0224
NM_176614.1	pregnancy-associated glycoprotein 2	*PAG2*	-2.28	0.0001

Supplementary Table 3.
List of differentially expressed transcripts between in vivo- (ET) and in vitro- (IVP) produced bovine blastocysts.

Representative Public ID	**Gene Title**	**Gene Symbol**	**Fold change**	***P*-value**
CB427000	Transcribed locus, moderately similar to NP_989434.1 gamma-adducin [Gallus gallus]	---	-4.56	0.0001
BF043546	S100 calcium binding protein A2	*S100A2*	-4.35	0.0001
CB171376	major allergen Equ c 1-like	*LOC783399*	-4.18	0.0020
CK953121	plastin 1	*PLS1*	-4.03	0.0008
BE752516	shisa homolog 2 (Xenopus laevis)	*SHISA2*	-3.92	0.0001
CB166446	nucleobindin 2	*NUCB2*	-3.85	0.0001
M31556.1	interferon tau	*IFNT2*	-3.76	0.0001
CK772448	tubulin, beta 2A	*TUBB2B*	-3.66	0.0003
CK955586	destrin (actin depolymerizing factor)	*DSTN*	-3.53	0.0010
AU278717	P antigen family, member 4 (prostate associated)	*PAGE4*	-3.47	0.0014
CK848316	palladin, cytoskeletal associated protein	*PALLD*	-3.46	0.0007
CK775121	heme binding protein 2	*HEBP2*	-3.42	0.0006
CK976192	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	*HMGCS1*	-3.41	0.0040

CK847756	actin, gamma 1	*ACTG1*	-3.36	0.0114
NM_174431.1	peroxiredoxin 1	*PRDX1*	-3.34	0.0094
CK848418	keratin 18	*KRT18*	-3.31	0.0006
CK950834	cyclin G1	*CCNG1*	-3.30	0.0002
NM_174409.2	osteoclast stimulating factor 1	*OSTF1*	-3.27	0.0025
CK974301	pituitary tumor-transforming 1 interacting protein	*PTTG1IP*	-3.25	0.0015
CK968451	glycyl-tRNA synthetase	*GARS*	-3.19	0.0001
CB170514	ribosomal protein L7	*RPL7*	-3.15	0.0153
CB165272	WD repeat domain 54	*WDR54*	-3.13	0.0016
CB436429	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	*MTHFD2*	-3.12	0.0002
CK961820	cytochrome P450, family 51, subfamily A, polypeptide 1	*CYP51A1*	-3.07	0.0012
NM_174062.2	ferritin, heavy polypeptide 1	*FTH1*	-3.03	0.0068
NM_174099.2	lactate dehydrogenase A	*LDHA*	-3.00	0.0001
CB171187	ATPase, Na+/K+ transporting, beta 3 polypeptide	*ATP1B3*	-2.96	0.0034
NM_174650.1	S100 calcium-binding protein A10	*S100A10*	-2.94	0.0004
CK776082	plasma membrane proteolipid (plasmolipin)	*PLLP*	-2.94	0.0001
CK969361	tumor protein, translationally-controlled 1	*TPT1*	-2.93	0.0051
CB461365	PREDICTED: Bos taurus uncharacterized LOC100850521 (LOC100850521), miscRNA	---	-2.93	0.0092
CK955677	keratin 19	*KRT19*	-2.93	0.0001
CK848695	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	*HSPA5*	-2.92	0.0018
CK780160	ribosomal protein S27-like	*RPS27L*	-2.91	0.0033
BM435101	zinc finger protein 36, C3H type-like 1	*ZFP36L1*	-2.91	0.0001
CK972401	chloride intracellular channel 1	*CLIC1*	-2.90	0.0170
BI680642	clathrin, heavy chain (Hc)	*CLTC*	-2.87	0.0199
CB443595	tumor protein, translationally-controlled 1	*TPT1*	-2.87	0.0186
AV615649	tetraspanin 6	*TSPAN6*	-2.83	0.0008
CB171354	epithelial cell adhesion molecule	*EPCAM*	-2.80	0.0029
CK966964	Protein tyrosine phosphatase type IVA, member 1	*PTP4A1*	-2.79	0.0165

CK948397	eukaryotic translation elongation factor 1 beta 2	*EEF1B*	-2.79	0.0159
CK848320	ornithine aminotransferase	*OAT*	-2.78	0.0185
CB168618	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	*GNB2L1*	-2.78	0.0050
CK849687	lectin, galactoside-binding, soluble, 3	*LGALS3*	-2.76	0.0169
AB032826.1	selenoprotein P, plasma, 1	*SEPP1*	-2.76	0.0017
NM_174149.1	ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c	*ATP6V0C*	-2.75	0.0058
CK728409	cornichon homolog 4 (Drosophila)	*CNIH4*	-2.74	0.0015
AF013064	ribosomal protein L6	*RPL6*	-2.74	0.0249
BM252020	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)	*DEGS1*	-2.73	0.0062
CK969361	tumor protein, translationally-controlled 1	*TPT1*	-2.73	0.0142
NM_174780.2	brain abundant, membrane attached signal protein 1	*BASP1*	-2.72	0.0156
CB172029	ribosomal protein L4	*RPL4*	-2.72	0.0117
X04851.1	clathrin, light chain (Lca)	*CLTA*	-2.71	0.0054
BF889659	RAB5-interacting protein	*RIP5*	-2.70	0.0098
BG358191	golgin A4	*GOLGA4*	-2.69	0.0069
CK969180	ribosomal protein L9	*RPL9*	-2.68	0.0151
CK770477	cysteine and glycine-rich protein 2	*CSRP2*	-2.67	0.0183
NM_173968.2	thioredoxin	*TXN*	-2.67	0.0024
CK977042	testis expressed 2	*TEX2*	-2.67	0.0005
AW315315	12525 MARC 2BOV Bos taurus cDNA 5-, mRNA sequence	---	-2.67	0.0213
NM_174315.2	fatty acid binding protein 5 (psoriasis-associated)	*FABP5*	-2.65	0.0001
BM929161	ribosomal protein L23	*RPL23*	-2.64	0.0109
NM_174689.1	ADP-ribosylation factor 4	*ARF4*	-2.61	0.0001
AB099060.1	ribosomal protein S2	*RPS2*	-2.60	0.0094
NM_173893.1	beta-2-microglobulin	*B2M*	-2.60	0.0078
CB166924	ribosomal protein L5	*RPL5*	-2.60	0.0093
CK948251	sorting nexin 2	*SNX2*	-2.59	0.0146
CK968996	sorbitol dehydrogenase	*SORD*	-2.59	0.0100

CK848675	AHNAK nucleoprotein	*AHNAK*	-2.58	0.0041
CK941764	transmembrane protein 14C	*TMEM14C*	-2.57	0.0074
CB168037	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	*SLC16A1*	-2.57	0.0060
NM_174147.2	plasminogen activator, urokinase	*PLAU*	-2.56	0.0023
CK953000	lactate dehydrogenase B	*LDHB*	-2.56	0.0357
CK952806	similar to ribosomal protein S6-like /// ribosomal protein S6	*LOC787914 /// RPS6*	-2.54	0.0137
CK975591	carboxymethylenebutenolidase homolog (Pseudomonas)	*CMBL*	-2.53	0.0001
BP103379	PREDICTED: Bos taurus uncharacterized LOC100847108 (LOC100847108), miscRNA	---	-2.52	0.0001
CK940634	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	*GNAI3*	-2.50	0.0030
CK778202	phosphatidylinositol transfer protein, alpha	*PITPNA*	-2.48	0.0002
BE750213	claudin 23	*CLDN23*	-2.48	0.0053
NM_174472.2	TIMP metallopeptidase inhibitor 2	*TIMP2*	-2.46	0.0001
CB171483	nucleosome assembly protein 1-like 1	*NAP1L1*	-2.46	0.0097
CK974042	ribosomal protein S3A	*RPS3A*	-2.46	0.0189
CB451042	eukaryotic translation initiation factor 1A, X-linked	*EIF1AX*	-2.45	0.0187
AV602127	eukaryotic translation elongation factor 1 alpha 1 /// similar to eukaryotic translation elongation factor 1 alpha 1 /// similar to eukaryotic translation elongation factor 1 alpha 1	*EEF1A1 /// LOC782989 /// LOC789867*	-2.45	0.0124
BP108852	ribosomal protein S18	*RPS18*	-2.44	0.0211
AW266900	fragile X mental retardation, autosomal homolog 1	*FXR1*	-2.44	0.0110
AW657942	Bos taurus cDNA clone IMAGE:7944277	---	-2.44	0.0350
CK770348	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	*CASK*	-2.44	0.0003
BP108127	ribosomal protein L27	*RPL27*	-2.43	0.0217
CK770030	ribosomal protein L11	*RPL11*	-2.42	0.0239
BM251913	ribosomal protein S14	*RPS14*	-2.42	0.0096

CB420281	desmoglein 2	*DSG2*	-2.42	0.0286
CB423230	CD48 molecule	*CD48*	-2.41	0.0288
CK770847	heme oxygenase (decycling) 1	*HMOX1*	-2.39	0.0009
NM_175784.2	annexin A1	*ANXA1*	-2.39	0.0219
CB425639	placenta-specific 8	*PLAC8*	-2.38	0.0053
NM_174760.2	ribosomal protein L10	*RPL10*	-2.38	0.0119
CB172030	cornichon homolog 4 (Drosophila)	*CNIH4*	-2.38	0.0001
BM483751	golgi reassembly stacking protein 2, 55kDa	*GORASP2*	-2.37	0.0021
CK953606	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	*CYP3A28*	-2.37	0.0426
CK957037	RAB11A, member RAS oncogene family	*RAB11A*	-2.37	0.0257
CK953368	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	*YWHAQ*	-2.37	0.0217
CB166882	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	*NPM1*	-2.36	0.0481
CK771078	gamma-inducible protein 30	*IFI30*	-2.36	0.0001
CB165323	phosphatidylethanolamine binding protein	*PEBP1*	-2.36	0.0269
NM_174806.2	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	*GOT2*	-2.36	0.0025
CB165391	actin related protein 2/3 complex, subunit 5, 16kDa	*ARPC5*	-2.36	0.0005
CK848538	heat shock 70kDa protein 4	*HSPA4*	-2.35	0.0304
BP105001	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	*PIP4K2A*	-2.34	0.0020
CK972263	translocation associated membrane protein 1	*TRAM1*	-2.34	0.0065
CK953351	hydroxysteroid (17-beta) dehydrogenase 11	*HSD17B11*	-2.34	0.0398
CA035980	ribosomal protein S23	*RPS23*	-2.34	0.0339
BE682987	core-binding factor, beta subunit	---	-2.34	0.0132
NM_175782.1	lectin, galactoside-binding, soluble, 1	*LGALS1*	-2.33	0.0041
CK941606	ribosomal protein L3	*Rpl3*	-2.33	0.0212
BE756166	quaking homolog, KH domain RNA binding (mouse)	---	-2.32	0.0025
CK848838	CAP, adenylate cyclase-associated protein 1 (yeast)	*CAP1*	-2.32	0.0061
CB424390	testis expressed 12	*TEX12*	-2.31	0.0019

CK945714	Obg-like ATPase 1	*OLA1*	-2.31	0.0196
CK845887	ATPase, H+ transporting, lysosomal V0 subunit a4	*ATP6V0A4*	-2.31	0.0034
CB167946	ribosomal protein S3	*RPS3*	-2.31	0.0108
NM_174241.2	annexin A8	*ANXA8*	-2.31	0.0039
CK978877	heterogeneous nuclear ribonucleoprotein A/B	*HNRNPAB*	-2.30	0.0221
CK956896	keratin 8	*KRT8*	-2.29	0.0017
CK945417	transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	*TCEB1*	-2.29	0.0090
CK953517	programmed cell death 6	*PDCD6*	-2.29	0.0088
BP107427	ribosomal protein L26	*RPL26*	-2.29	0.0224
CB537825	PREDICTED: Bos taurus uncharacterized LOC100848375 (LOC100848375), miscRNA	---	-2.28	0.0001
CK979098	NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae)	*NFU1*	-2.28	0.0054
CK770170	Asparaginase like 1	*ASRGL1*	-2.28	0.0212
CB166901	phosphoserine aminotransferase 1	*PSAT1*	-2.28	0.0005
CK941902	oxysterol binding protein-like 1A	*OSBPL1A*	-2.28	0.0241
CB449605	chloride intracellular channel 4	*CLIC4*	-2.28	0.0059
CK775789	transmembrane protein 97	*TMEM97*	-2.28	0.0215
M81190.1	desmocollin 2	*DSC2*	-2.28	0.0066
CB433697	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	*PSMD14*	-2.27	0.0318
CK952849	similar to ribosomal protein S4, X-linked X /// similar to ribosomal protein S4, X-linked X /// similar to ribosomal protein S4, X-linked X /// ribosomal protein S4, X-linked /// ribosomal protein S4, Y-linked 1 /// ribosomal protein S4, Y-linked 2	*LOC781612 /// LOC783463 /// LOC784060 /// RPS4X /// RPS4Y1 /// RPS4Y2*	-2.27	0.0218
BI538866	ADAM metallopeptidase domain 9 (meltrin gamma)	*ADAM9*	-2.27	0.0042
BE751337	acyl-CoA synthetase long-chain family member 3	*ACSL3*	-2.27	0.0075
CK848926	protein disulfide isomerase family A, member 6	*PDIA6*	-2.26	0.0006
CB533161	transmembrane BAX inhibitor motif containing 6	*TMBIM6*	-2.26	0.0041

NM_175813.1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	*EIF2S1*	-2.25	0.0174
CB533458	synaptophysin-like 1	*SYPL1*	-2.25	0.0029
CB169082	ribosomal protein, large, P0	*RPLP0*	-2.25	0.0226
D00467.1	ferredoxin 1	*FDX1*	-2.24	0.0046
CB531064	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	*ST13*	-2.24	0.0376
CK974513	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	*PSMD11*	-2.24	0.0192
NM_176614.1	pregnancy-associated glycoprotein 2	*PAG2*	-2.24	0.0001
CK779560	UMC-bend_0A02-013-e05 Day 8 Uterus bend Bos taurus cDNA 3-, mRNA sequence	---	-2.24	0.0008
AW485507	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	*EIF2S3*	-2.24	0.0298
CK973336	ribosomal protein, large, P0	*RPLP0*	-2.24	0.0019
CK966418	4081208 BARC 9BOV Bos taurus cDNA clone 9BOV1_A09 3-, mRNA sequence	---	-2.23	0.0009
CK972818	Parkinson disease (autosomal recessive, early onset) 7	*PARK7*	-2.23	0.0041
BI539096	KH domain containing, RNA binding, signal transduction associated 1	*KHDRBS1*	-2.22	0.0003
BM366524	family with sequence similarity 32, member A	*FAM32A*	-2.21	0.0188
BE236720	capping protein (actin filament) muscle Z-line, alpha 1	*CAPZA1*	-2.21	0.0167
NM_175780.1	myosin, light chain 6, alkali, smooth muscle and non-muscle	*MYL6*	-2.21	0.0200
CK728106	SUB1 homolog (S. cerevisiae)	*SUB1*	-2.21	0.0078
NM_174049.1	enolase 1, (alpha)	*ENO1*	-2.21	0.0035
CB420617	solute carrier family 13 (sodium/sulfate symporters), member 4	*SLC13A4*	-2.21	0.0006
NM_175814.2	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	*SDHC*	-2.21	0.0157
CK961971	ribosomal protein L17	*RPL17*	-2.21	0.0448
NM_205797.1	similar to 60S ribosomal protein L12 /// ribosomal protein L12	*LOC784762 /// RPL12*	-2.21	0.0308
CK774588	tight junction protein 1 (zona occludens 1)	*TJP1*	-2.20	0.0125
CB165372	similar to MORF-related gene 15	*LOC785568*	-2.20	0.0459

CK948193	integral membrane protein 2B	*ITM2B*	-2.19	0.0104
CK849608	eukaryotic translation elongation factor 2	*EEF2*	-2.19	0.0486
CB464010	mitochondrial carrier homolog 1 (C. elegans)	*MTCH1*	-2.18	0.0176
CK969113	ornithine decarboxylase antizyme 1	*OAZ1*	-2.18	0.0295
BM251691	ribosomal protein L27a	*RPL27A*	-2.18	0.0243
AY528251.1	ribosomal protein S12	*RPS12*	-2.18	0.0342
CB445831	quinone oxidoreductase-like protein 2-like	*LOC100299281*	-2.17	0.0002
CB463704	wntless homolog (Drosophila)	*WLS*	-2.17	0.0009
CB448765	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	*CLN8*	-2.17	0.0003
CB535820	SMAD specific E3 ubiquitin protein ligase 2	*SMURF2*	-2.17	0.0073
CK849793	sequestosome 1	*SQSTM1*	-2.16	0.0456
CK773855	eukaryotic translation elongation factor 1 gamma	*EEF1G*	-2.16	0.0024
CB167065	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	*ITGB1*	-2.16	0.0412
BM255343	myosin, light chain 7, regulatory	*MYL7*	-2.16	0.0001
CK961338	SEC31 homolog A (S. cerevisiae)	*SEC31A*	-2.15	0.0097
CK770475	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	*HIF1A*	-2.15	0.0005
NM_174217.1	ezrin	*EZR*	-2.14	0.0136
CK770613	filamin binding LIM protein 1	*FBLIM1*	-2.14	0.0004
NM_177516.1	glutathione S-transferase pi 1	*GSTP1*	-2.13	0.0014
CK769429	lysosomal-associated membrane protein 1	*LAMP1*	-2.13	0.0010
CK968736	ribosomal protein S8	*RPS8*	-2.13	0.0465
CB530156	calmodulin 1 (phosphorylase kinase, delta) /// calmodulin 3 (phosphorylase kinase, delta)	*CALM1 /// CALM3*	-2.13	0.0028
CB433764	Kruppel-like factor 6	*KLF6*	-2.12	0.0030
CK848693	mesoderm development candidate 2	*MESDC2*	-2.12	0.0046
CK848313	eukaryotic translation initiation factor 4B	*EIF4B*	-2.12	0.0209
CB171490	similar to Coiled-coil-helix-coiled-coil-helix domain Transcribed	---	-2.12	0.0100

	locus, strongly similar to XP_343484.1 PREDICTED: dystroglycan 1 (dystrophin-associated glycoprotein 1) [Rattus norvegicus]			
CB451437	transmembrane 9 superfamily member 2	*TM9SF2*	-2.12	0.0285
CK965736	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa	*VAPA*	-2.11	0.0395
CB170586	tumor-associated calcium signal transducer 2	*TACSTD2*	-2.11	0.0080
NM_174281.2	coatomer protein complex, subunit beta 2 (beta prime)	*COPB2*	-2.11	0.0325
CB456571	RAB1A, member RAS oncogene family	*RAB1A*	-2.11	0.0161
BM435937	polymerase (DNA-directed), epsilon 4 (p12 subunit)	*POLE4*	-2.11	0.0038
CK969757	ubiquitin carboxyl-terminal hydrolase L5	*UCHL5*	-2.11	0.0146
CB166320	splicing factor, arginine/serine-rich 3	*SFRS3*	-2.10	0.0419
BE750474	NCK adaptor protein 1	*NCK1*	-2.10	0.0006
NM_174729.2	voltage-dependent anion channel 1 pseudogene 5	*VDAC1P5*	-2.10	0.0025
CK949239	signal peptidase complex subunit 2 homolog (S. cerevisiae)	*SPCS2*	-2.10	0.0209
CK773259	bifunctional apoptosis regulator	*BFAR*	-2.10	0.0341
CK849836	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	*STT3B*	-2.10	0.0001
CB421885	proteasome (prosome, macropain) subunit, beta type, 2	*PSMB2*	-2.09	0.0063
CK848736	peptidase (mitochondrial processing) beta	*PMPCB*	-2.09	0.0135
CB440375	interleukin-33	*IL33*	-2.09	0.0143
CK849088	MHC class I antigen /// MHC Class I JSP.1 /// hypothetical protein 100125016	*BOLA-N /// JSP.1 /// LOC100125916*	-2.09	0.0100
CK948152	similar to Protein-S-isoprenylcysteine O-methyltransferase (Isoprenylcysteine carboxylmethyltransferase) (Prenylcysteine carboxyl methyltransferase) (pcCMT) (Prenylated protein carboxyl methyltransferase) (PPMT)	---	-2.09	0.0002
CB459236	desmoplakin	*DSP*	-2.09	0.0081
NM_174778.1	ribosomal protein S27a	*RPS27A*	-2.08	0.0335
NM_174749.2	peroxiredoxin 5	*PRDX5*	-2.08	0.0023

CB455141	actin related protein 2/3 complex, subunit 2, 34kDa /// similar to actin related protein 2/3 complex subunit 2	*ARPC2 /// LOC785054*	-2.08	0.0324
BE685292	podoplanin	*PDPN*	-2.08	0.0204
CB444452	p21 protein (Cdc42/Rac)-activated kinase 1	*PAK1*	-2.08	0.0003
CK847195	stratifin	*SFN*	-2.08	0.0003
AB099060.1	ribosomal protein S2	*RPS2*	-2.07	0.0226
CK951179	CSE1 chromosome segregation 1-like (yeast)	*CSE1L*	-2.07	0.0124
CB531601	eukaryotic translation initiation factor 1	*EIF1*	-2.07	0.0315
AV601221	ribosomal protein L8	*RPL8*	-2.06	0.0247
CB172782	SFT2 domain containing 1	*SFT2D1*	-2.06	0.0158
CK848915	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	*MLH1*	-2.05	0.0017
CK769520	oligosaccharyltransferase complex subunit	*OSTC*	-2.05	0.0107
AV618700	WW domain-binding protein 2	*WBP2*	-2.05	0.0271
BE478318	Protein FAM177A1	*FAM177A1*	-2.05	0.0193
CK849191	X-box binding protein pseudogene 1	*XBPP1*	-2.05	0.0004
NM_174568.1	poly(A) binding protein, cytoplasmic 1	*PABPC1*	-2.04	0.0214
NM_174455.2	ribosomal protein L24	*RPL24*	-2.04	0.0138
AU276080	ADP-ribosylation factor-like protein 8A	---	-2.04	0.0006
CK967649	zinc finger protein 207	*ZNF207*	-2.04	0.0094
CK849180	translocase of outer mitochondrial membrane 20 homolog (yeast)	*TOMM20*	-2.04	0.0491
AV610198	prolyl 4-hydroxylase, beta polypeptide	*P4HB*	-2.04	0.0236
CK964985	annexin A3	*ANXA3*	-2.04	0.0012
CB168642	chromosome 20 open reading frame 30 ortholog	*C13H20orf30*	-2.04	0.0047
CK948574	beta-2-microglobulin	*B2M*	-2.03	0.0037
BP101535	phosphatidylinositol binding clathrin assembly protein	*PICALM*	-2.03	0.0345
NM_174333.2	protein disulfide isomerase family A, member 3	*PDIA3*	-2.03	0.0050
NM_174711.2	casein kinase 1, alpha 1	*CSNK1A1*	-2.03	0.0045
BP108263	secreted seminal-vesicle Ly-6 protein 1	*SSLP1*	-2.03	0.0075
CB533579	RAB10, member RAS oncogene family	*RAB10*	-2.02	0.0133
BM433128	FK506 binding protein 9, 63 kDa	---	-2.02	0.0027

CK975976	death-associated protein	*DAP*	-2.02	0.0016
AY342429.1	CD46 molecule, complement regulatory protein	*CD46*	-2.01	0.0397
NM_174226.2	ARP3 actin-related protein 3 homolog (yeast)	*ACTR3*	-2.01	0.0350
CK974450	basic transcription factor 3	*BTF3*	-2.01	0.0154
CK776121	NSA2 ribosome biogenesis homolog (S. cerevisiae)	*NSA2*	-2.00	0.0197
CB439434	similar to testis derived transcript /// testis derived transcript (3 LIM domains)	*LOC789240 /// TES*	-2.00	0.0007
CK847310	syntaxin 8	*STX8*	-2.00	0.0072
CB461909	ATX1 antioxidant protein 1 homolog (yeast)	*ATOX1*	-2.00	0.0001
AV618619	presenilin 1	*PSEN1*	-2.00	0.0280
CK948244	malate dehydrogenase 1, NAD (soluble)	*MDH1*	-1.99	0.0361
AV608010	laminin, gamma 1 (formerly LAMB2)	*LAMC1*	-1.99	0.0005
CK774111	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	*PSMD10*	-1.99	0.0012
BM104814	dynein, light chain, Tctex-type 3 /// similar to TCTE1L	*DYNLT3 /// LOC782745*	-1.99	0.0224
CB463753	ribosomal protein S16	*RPS16*	-1.99	0.0449
CB431775	Bos taurus uncharacterized LOC100847427 (LOC100847427), miscRNA	---	-1.99	0.0008
BE722892	F-box protein 7	*FBXO7*	-1.99	0.0181
AU276294	3-hydroxy-3-methylglutaryl-CoA reductase	*HMGCR*	-1.99	0.0479
BP108845	ribosomal protein S21	*RPS21*	-1.98	0.0045
CB165258	sterol-C4-methyl oxidase-like	*SC4MOL*	-1.98	0.0074
CK977244	ribosomal protein L19	*RPL19*	-1.98	0.0100
CK846611	tumor susceptibility gene 101	*TSG101*	-1.98	0.0108
CK847264	lysosomal protein transmembrane 4 alpha	*LAPTM4A*	-1.97	0.0177
AY241933.1	stearoyl-CoA desaturase (delta-9-desaturase)	*SCD*	-1.97	0.0060
CB446456	thiosulfate sulfurtransferase (rhodanese)-like domain containing 1	*TSTD1*	-1.97	0.0285
NM_175803.2	tubulin folding cofactor A	*TBCA*	-1.97	0.0051
CK974463	dynein, cytoplasmic 1, intermediate chain 2	*DYNC1I2*	-1.97	0.0410
CB431074	ribosomal protein S25 /// similar to mCG10725	*RPS25 /// RPS25*	-1.96	0.0361

CK848228	nuclear factor (erythroid-derived 2)-like 2	*NFE2L2*	-1.96	0.0208
CB456714	CTTNBP2 N-terminal-like protein	---	-1.96	0.0296
CB432286	asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog (S. cerevisiae)	*ALG9*	-1.96	0.0003
NM_174343.2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	*HSD3B1*	-1.96	0.0003
BP108713	ribosomal protein L23a	*RPL23A*	-1.95	0.0348
CK848474	myoferlin	*MYOF*	-1.94	0.0068
CK979703	plakophilin 2	*PKP2*	-1.94	0.0357
CB464066	clathrin, heavy chain (Hc)	*CLTC*	-1.94	0.0424
BE684355	protein tyrosine phosphatase, receptor type, K	*PTPRK*	-1.94	0.0014
CK961552	EFR3 homolog A (S. cerevisiae)	*EFR3A*	-1.94	0.0079
CK975718	nuclear receptor coactivator 4	*NCOA4*	-1.93	0.0137
NM_174810.2	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1	*ATP6V1E1*	-1.93	0.0082
CK981091	signal sequence receptor, alpha	*SSR1*	-1.93	0.0047
AU275302	GLI pathogenesis-related 2	*GLIPR2*	-1.92	0.0302
NM_174340.2	histidine triad nucleotide binding protein 2	*HINT2*	-1.92	0.0062
CK773920	protease, serine, 23	*PRSS23*	-1.92	0.0005
CK963545	endothelial PAS domain protein 1	*EPAS1*	-1.92	0.0119
CK974007	mesencephalic astrocyte-derived neurotrophic factor	*MANF*	-1.92	0.0041
BP108915	dynein, light chain, LC8-type 1	*DYNLL1*	-1.92	0.0093
CK848969	Transcription elongation factor A (SII)-like 8	*TCEAL8*	-1.92	0.0002
AV602184	tubulin, alpha 1a /// tubulin, alpha 1b /// tubulin, alpha 1c	*TUBA1A /// TUBA1B /// TUBA1C*	-1.91	0.0069
BE752394	ribosomal protein L31	*RPL31*	-1.91	0.0472
BE666389	stress-associated endoplasmic reticulum protein 1	*SERP1*	-1.91	0.0013
CK951982	general transcription factor IIE, polypeptide 2, beta 34kDa	*GTF2E2*	-1.91	0.0443
AU275257	ribosomal protein L18	*RPL18*	-1.91	0.0137
CK953663	ribosomal protein SA	*RPSA*	-1.90	0.0065

CB451598	chaperonin containing TCP1, subunit 4 (delta)	*CCT4*	-1.90	0.0492
CB430170	606040 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.90	0.0162
CK960868	thioesterase superfamily member 4	*THEM4*	-1.90	0.0006
CK963094	hypothetical protein MGC127538	*MGC127538*	-1.90	0.0392
AW311862	desmocollin 2	---	-1.90	0.0002
BP106980	aldo-keto reductase family 1, member B1 (aldose reductase)	*AKR1B1*	-1.89	0.0036
BF776610	7-dehydrocholesterol reductase	*DHCR7*	-1.89	0.0144
CB467891	polymerase (RNA) I polypeptide D, 16kDa	*POLR1D*	-1.89	0.0021
BE684608	protease, serine, 8	*PRSS8*	-1.89	0.0021
NM_175800.2	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	*NDUFS4*	-1.89	0.0184
CB166358	lysosomal-associated membrane protein 2	*LAMP2*	-1.89	0.0147
CB450657	transmembrane protein 85	*TMEM85*	-1.88	0.0034
CK777018	choline phosphotransferase 1	*CHPT1*	-1.88	0.0004
BF043332	ras suppressor protein 1	*RSU1*	-1.88	0.0028
CK947713	transmembrane protein 49	*TMEM49*	-1.88	0.0089
NM_177520.2	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	*MGAT4A*	-1.88	0.0028
CK846717	proteasome (prosome, macropain) 26S subunit, ATPase, 6	*PSMC6*	-1.88	0.0383
BP107757	ribosomal protein S20	*RPS20*	-1.88	0.0088
AV610889	glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	-1.87	0.0030
CK769676	mitochondrial ribosomal protein L45	*MRPL45*	-1.87	0.0009
CK955846	mitochondrial ribosomal protein L3	*MRPL3*	-1.87	0.0136
AV617586	fermitin family homolog 2 (Drosophila)	*FERMT2*	-1.87	0.0374
CK958502	filamin A interacting protein 1-like	*FILIP1L*	-1.87	0.0255
BP103163	BTB (POZ) domain containing 1	*BTBD1*	-1.86	0.0290
CK974428	chromosome 11 open reading frame 73 ortholog	*C29H11orf73*	-1.86	0.0233
CK771035	interferon regulatory factor 5	*IRF5*	-1.86	0.0012
CK848932	fucosidase, alpha-L- 1, tissue	*FUCA1*	-1.85	0.0019
AU098162	AU098162 Cloned bovine fetus cDNA Bos taurus cDNA clone	---	-1.85	0.0129

	Cln454 3-, mRNA sequence			
CK778646	proteasome (prosome, macropain) assembly chaperone 1	*PSMG1*	-1.85	0.0243
CK775998	latrophilin 2	*LPHN2*	-1.85	0.0030
NM_174465.2	SEC11 homolog A (S. cerevisiae)	*SEC11A*	-1.85	0.0140
CB172516	hypothetical LOC513740	*LOC513740*	-1.85	0.0313
CK943646	arsenic (+3 oxidation state) methyltransferase	*AS3MT*	-1.85	0.0004
NM_174445.2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	*PTGS2*	-1.85	0.0046
CK975949	quinoid dihydropteridine reductase	*QDPR*	-1.85	0.0423
CK772180	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	*ATP6V0D2*	-1.84	0.0310
CK952626	adaptor-related protein complex 3, sigma 1 subunit	*AP3S1*	-1.84	0.0153
CB468257	ataxin 2	*ATXN2*	-1.83	0.0408
CK948086	vesicle-associated membrane protein 3 (cellubrevin)	*VAMP3*	-1.83	0.0407
CK950056	deoxyribonuclease I-like 3	*DNASE1L3*	-1.83	0.0431
BF653531	vacuolar protein sorting 41 homolog (S. cerevisiae)	*VPS41*	-1.83	0.0009
NP_776577.1	peptidylprolyl isomerase B (cyclophilin B)	*PPIB*	-1.83	0.0038
CK950519	dehydrogenase E1 and transketolase domain containing 1	*DHTKD1*	-1.82	0.0090
CK847287	ADP-ribosylation factor 4 /// similar to ADP-ribosylation factor 4	*ARF4 /// LOC614581*	-1.82	0.0206
CK977019	mitotic checkpoint component Mad2 /// MAD2 mitotic arrest deficient-like 1 (yeast)	*MAD2 /// MAD2L1*	-1.82	0.0139
BE667214	metadherin	*MTDH*	-1.82	0.0419
CK774104	mitochondrial ribosomal protein L19	*MRPL19*	-1.82	0.0027
CK846906	DnaJ (Hsp40) homolog, subfamily B, member 1	*DNAJB1*	-1.82	0.0033
CK948151	ribophorin II	*RPN2*	-1.81	0.0324
BI541888	hypothetical protein LOC532603	*LOC532603*	-1.81	0.0001
CK960514	eukaryotic translation initiation factor 3, subunit F	*EIF3F*	-1.81	0.0310
AU275462	collagen, type XII, alpha 1	*COL12A1*	-1.81	0.0017
NM_175831.2	cytochrome c oxidase subunit VIIc	*COX7C*	-1.81	0.0152
NM_201527.1	superoxide dismutase 2, mitochondrial	*SOD2*	-1.80	0.0445

CK957676	ARP2 actin-related protein 2 homolog (yeast)	*ACTR2*	-1.80	0.0249
CK948142	chromosome 10 open reading frame 84 ortholog	*C26H10orf84*	-1.79	0.0346
CK772973	dicarbonyl/L-xylulose reductase	*DCXR*	-1.79	0.0346
CB464064	superoxide dismutase 1, soluble	*SOD1*	-1.79	0.0010
BP103554	phosphatase, orphan 2	*PHOSPHO2*	-1.79	0.0216
BP104582	adducin 3 (gamma)	---	-1.78	0.0473
CK954454	3-ketodihydrosphingosine reductase	*KDSR*	-1.78	0.0093
CK980844	beta-carotene oxygenase 2	*BCO2*	-1.78	0.0014
CK951345	PREDICTED: yorkie homolog	---	-1.78	0.0260
CB434763	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	*YWHAG*	-1.78	0.0480
CK973814	solute carrier family 44, member 1	*SLC44A1*	-1.78	0.0011
CB531775	CDV3 homolog (mouse)	*CDV3*	-1.78	0.0376
CB169092	similar to Uncharacterized protein C1orf175 homolog	---	-1.78	0.0229
M13236.1	guanine nucleotide binding protein (G protein), beta polypeptide 1	*GNB1*	-1.78	0.0033
CB172231	IMP (inosine monophosphate) dehydrogenase 2	*IMPDH2*	-1.77	0.0222
CK972144	N-acylsphingosine amidohydrolase (acid ceramidase) 1	*ASAH1*	-1.77	0.0054
CB166154	ralA binding protein 1	*RALBP1*	-1.77	0.0137
CK848295	adaptor-related protein complex 2, beta 1 subunit	*AP2B1*	-1.77	0.0056
CK775888	sepiapterin reductase (7,8-dihydrobiopterin:NADP+ oxidoreductase)	*SPR*	-1.77	0.0051
CB447825	Anillin, actin binding protein	*ANLN*	-1.77	0.0230
CK847914	MPV17 mitochondrial membrane protein-like 2	*MPV17L2*	-1.77	0.0010
CK770918	transcription elongation factor A (SII)-like 4	*TCEAL4*	-1.76	0.0039
CK940683	replication protein A1, 70kDa	*RPA1*	-1.76	0.0003
CF764324	coiled-coil domain containing 90A	*CCDC90A*	-1.76	0.0037
CK980929	N-acetylgalactosaminidase, alpha-	*NAGA*	-1.75	0.0042
BM255214	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30kDa	*TAF10*	-1.75	0.0150
CB432337	sorting nexin 4	*SNX4*	-1.75	0.0198

AV607592	actin, beta	*ACTB*	-1.75	0.0107
NM_174080.2	ubiquitin-conjugating enzyme E2K (UBC1 homolog, yeast)	*UBE2K*	-1.75	0.0328
CB420377	catenin, beta interacting protein 1	*CTNNBIP1*	-1.74	0.0027
CK948245	mitogen-activated protein kinase 14	*MAPK14*	-1.74	0.0089
BM445534	PREDICTED: Bos taurus uncharacterized LOC100849050 (LOC100849050), miscRNA	---	-1.74	0.0017
CK945640	FAT tumor suppressor homolog 1 (Drosophila)	*FAT1*	-1.74	0.0165
CK849264	phosphoglycerate kinase 1	*PGK1*	-1.74	0.0351
AW447102	grancalcin, EF-hand calcium binding protein	---	-1.74	0.0304
CB424513	ATP-binding cassette, sub-family B (MDR/TAP), member 6	*ABCB6*	-1.74	0.0040
CK946141	hypothetical protein LOC510320	*LOC510320*	-1.74	0.0223
CB170194	trinucleotide repeat containing 6B	*TNRC6B*	-1.73	0.0043
CK849599	YME1-like 1 (S. cerevisiae)	*YME1L1*	-1.73	0.0104
NM_174384.2	lysyl oxidase-like 4	*LOXL4*	-1.73	0.0010
AV593983	similar to Zinc finger protein 330 /// zinc finger protein 330	*LOC787714 /// ZNF330*	-1.73	0.0381
CK775309	protein phosphatase 2, catalytic subunit, beta isozyme	*PPP2CB*	-1.73	0.0471
CB461923	histone-lysine N-methyltransferase SETMAR	---	-1.73	0.0124
BE667009	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	*B3GNT2*	-1.73	0.0070
CK950804	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	*KPNA2*	-1.73	0.0360
CK963170	ribosomal protein L7a	*RPL7A*	-1.73	0.0322
CB427169	SH3-domain binding protein 5 (BTK-associated)	*SH3BP5*	-1.73	0.0045
AV610889	glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	-1.73	0.0048
CK981095	desmocollin 2	---	-1.73	0.0019
CK971474	Ras homolog enriched in brain	*RHEB*	-1.73	0.0224
CB433327	609685 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.72	0.0098
NM_174163.2	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	*RAC1*	-1.72	0.0148
BE681760	kinectin 1 (kinesin receptor)	*KTN1*	-1.72	0.0171
BI849971	phospholysine phosphohistidine inorganic pyrophosphate	*LHPP*	-1.72	0.0067

	phosphatase			
CB535095	SH3 domain containing, Ysc84-like 1 (S. cerevisiae)	*SH3YL1*	-1.72	0.0014
NM_174830.1	eukaryotic translation initiation factor 6	*EIF6*	-1.72	0.0248
CB464654	inositol monophosphatase domain containing 1	*IMPAD1*	-1.72	0.0073
CK773530	N-terminal asparagine amidase	*NTAN1*	-1.72	0.0003
AV610144	annexin A2	*ANXA2*	-1.72	0.0105
NM_174792.2	ferritin, light polypeptide	*FTL*	-1.71	0.0003
CK950694	TSC22 domain family, member 1	*TSC22D1*	-1.71	0.0167
CK848754	melanoma antigen family D, 2	*MAGED2*	-1.70	0.0035
CK777001	integrator complex subunit 6	*INTS6*	-1.70	0.0344
BM257007	chromatin modifying protein 5	*CHMP5*	-1.70	0.0096
AV615731	COMM domain containing 8	*COMMD8*	-1.70	0.0119
CK971190	ribosomal protein L10a	*RPL10A*	-1.70	0.0155
NM_174821.2	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	*SERPING1*	-1.70	0.0032
CB420458	ADP-ribosylhydrolase like 2	*ADPRHL2*	-1.70	0.0312
CK958930	spastic paraplegia 20 (Troyer syndrome)	*SPG20*	-1.70	0.0011
CK954398	catalase	*CAT*	-1.70	0.0079
BE757079	CDC42 small effector 2	*CDC42SE2*	-1.69	0.0093
CK772515	claudin 6	*CLDN6*	-1.69	0.0339
CK770196	mitochondrial ribosomal protein L16	*MRPL16*	-1.69	0.0110
CB530449	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	*SLC11A2*	-1.69	0.0317
CK774881	single stranded DNA binding protein 3	*SSBP3*	-1.69	0.0169
NM_173900.2	CD9 molecule	*CD9*	-1.69	0.0309
CK774530	similar to Prion-like-(Q/N-rich)-domain-bearing protein family member (pqn-75)	*LOC524974*	-1.69	0.0264
M22559.1	ATPase inhibitory factor 1	*ATPIF1*	-1.69	0.0434
CK948267	KIAA0174	*IST1*	-1.69	0.0041
BE753186	carnosine synthase 1	*CARNS1*	-1.69	0.0484
BE664930	152833 MARC 4BOV Bos taurus cDNA 5-, mRNA sequence	---	-1.69	0.0058

BM252109	LIM domain only 4	*LMO4*	-1.69	0.0003
CB531088	unc-50 homolog (C. elegans)	*UNC50*	-1.69	0.0001
BM251302	growth hormone inducible transmembrane protein	*GHITM*	-1.68	0.0061
CB535077	SH3-domain kinase binding protein 1	*SH3KBP1*	-1.68	0.0062
BM364070	zinc finger, RAN-binding domain containing 2	*ZRANB2*	-1.68	0.0294
CK770852	solute carrier family 35 (CMP-sialic acid transporter), member A1	*SLC35A1*	-1.68	0.0002
AV616294	ribosomal protein L34	*RPL34*	-1.68	0.0215
CK848797	sterol carrier protein 2	*SCP2*	-1.68	0.0019
CK770586	nidogen 2 (osteonidogen)	*NID2*	-1.68	0.0107
CK846622	tumor protein D52	*TPD52*	-1.67	0.0096
AV590436	JNK1/MAPK8-associated membrane protein	*JKAMP*	-1.67	0.0050
BM430362	CD164 molecule, sialomucin	*CD164*	-1.67	0.0063
CK954456	v-ral simian leukemia viral oncogene homolog A (ras related)	*RALA*	-1.67	0.0082
CB460291	sorting nexin 6	*SNX6*	-1.67	0.0398
CK848993	thioredoxin domain containing 5 (endoplasmic reticulum)	*TXNDC5*	-1.67	0.0029
AW356461	major facilitator superfamily domain containing 1	*MFSD1*	-1.67	0.0084
NM_174212.1	UDP-glucose pyrophosphorylase 2	*UGP2*	-1.67	0.0327
CK771210	PDZ and LIM domain 1	*PDLIM1*	-1.66	0.0203
CK974440	amyloid beta (A4) precursor-like protein 2	*APLP2*	-1.66	0.0084
AV597296	phospholipase A2, group XV	*PLA2G15*	-1.66	0.0094
CK848545	insulin-like growth factor binding protein 7	*IGFBP7*	-1.66	0.0008
BE749971	Mannosidase, alpha, class 2A, member 1	*MAN2A1*	-1.66	0.0062
BE668288	Sec61 gamma subunit	*SEC61G*	-1.66	0.0110
NM_174222.2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	*TAP2*	-1.66	0.0009
CB531166	glia maturation factor, beta	---	-1.66	0.0401
BE237565	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	*SUGT1*	-1.66	0.0145
CB443641	valosin containing protein (p97)/p47 complex interacting protein 1	*VCPIP1*	-1.65	0.0481
CK846024	multiple coagulation factor deficiency 2	*MCFD2*	-1.65	0.0004
CK973552	4104251 BARC 9BOV Bos taurus cDNA clone 9BOV28_A10 3-, mRNA sequence	---	-1.65	0.0001

CK973241	amino-terminal enhancer of split	*AES*	-1.65	0.0008
CK778182	similar to Nitric oxide-inducible gene protein	*LOC618360*	-1.65	0.0171
CB169572	proteasome (prosome, macropain) subunit, beta type, 6	*PSMB6*	-1.64	0.0062
CB461755	aspartylglucosaminidase	*AGA*	-1.64	0.0003
CB169181	transportin 1	*TNPO1*	-1.64	0.0367
CK849512	CD63 molecule	*CD63*	-1.64	0.0058
AW656252	108398 MARC 1BOV Bos taurus cDNA 5-, mRNA sequence	---	-1.64	0.0014
NM_174770.2	glutathione peroxidase 4 (phospholipid hydroperoxidase)	*GPX4*	-1.64	0.0001
CK975764	synovial sarcoma translocation, chromosome 18	*SS18*	-1.64	0.0030
CK977771	transgelin 2	*TAGLN2*	-1.63	0.0330
CK774887	transmembrane emp24 protein transport domain containing 4	*TMED4*	-1.63	0.0365
CK983051	PREDICTED: Bos taurus uncharacterized LOC100850521 (LOC100850521), miscRNA	---	-1.63	0.0325
CB170168	SAR1 homolog A (S. cerevisiae)	---	-1.63	0.0366
CK965599	esterase D	*ESD*	-1.63	0.0097
CB433424	609787 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.63	0.0137
BI774070	ubiquitin specific peptidase 9, X-linked	*USP9X*	-1.63	0.0171
AU278311	solute carrier family 16, member 13 (monocarboxylic acid transporter 13)	*SLC16A13*	-1.63	0.0023
CK969061	nuclear casein kinase and cyclin-dependent kinase substrate 1	*NUCKS1*	-1.63	0.0035
CB456402	TBC1 domain family, member 20	*TBC1D20*	-1.62	0.0080
CK777681	KIAA1715	*KIAA1715*	-1.62	0.0292
CK971624	general transcription factor IIB /// similar to Transcription initiation factor IIB (General transcription factor TFIIB) (S300-II)	*GTF2B /// LOC786656*	-1.62	0.0338
BG358861	BOVMS1-002-Q1-E1-E7 Monsanto bovine skeletal muscle cDNA library BOVMS1 Bos taurus cDNA clone BOVMS1-002-Q1-E1-E7 5-, mRNA sequence	---	-1.62	0.0012
CB452134	SLAIN motif family, member 2	*SLAIN2*	-1.62	0.0044
AV594069	ribosomal protein L36	*RPL36*	-1.62	0.0323
CK849138	zinc finger protein 75D	*ZNF75D*	-1.62	0.0117

CK770335	PRELI domain containing 1	*PRELID1*	-1.62	0.0041
CK972445	similar to RNA-binding protein 35A (RNA-binding motif protein 35A)	*LOC788414*	-1.62	0.0273
CK967765	amyloid beta (A4) precursor protein	*APP*	-1.62	0.0109
CK941870	4065282 BARC 10BOV Bos taurus cDNA clone 10BOV12_A19 3-, mRNA sequence	---	-1.62	0.0142
CK975531	biliverdin reductase A	*BLVRA*	-1.61	0.0161
BP109178	SHC (Src homology 2 domain containing) transforming protein 1	*SHC1*	-1.61	0.0177
CK848549	immediate early response 3 interacting protein 1	*IER3IP1*	-1.61	0.0335
NM_174190.2	supervillin	*SVIL*	-1.61	0.0103
CK771661	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	*KDELR3*	-1.61	0.0023
CK959800	synaptosomal-associated protein, 23kDa	*SNAP23*	-1.61	0.0395
CB167910	pelota homolog (Drosophila)	*PELO*	-1.61	0.0009
CK970588	transmembrane protein C10orf57 homolog	*C28H10orf57*	-1.61	0.0076
NM_174152.2	peptidylprolyl isomerase B (cyclophilin B)	*PPIB*	-1.61	0.0007
CK957293	chromosome 11 open reading frame 75 ortholog /// similar to MGC133535 protein	*C29H11orf75 /// LOC786469*	-1.61	0.0029
CB465159	proteasome (prosome, macropain) subunit, alpha type, 5	*PSMA5*	-1.61	0.0224
AV601291	eukaryotic translation initiation factor 3, subunit E	*EIF3E*	-1.61	0.0265
CB166013	acyl-CoA binding domain containing 6	*ACBD6*	-1.60	0.0053
CK846911	acyl-CoA synthetase long-chain family member 6	*ACSL6*	-1.60	0.0139
CK777259	proline/serine-rich coiled-coil 1	*PSRC1*	-1.60	0.0063
CB430119	prefoldin subunit 1	*PFDN1*	-1.60	0.0168
CK771718	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	*DPM1*	-1.60	0.0074
CB166009	iron-sulfur cluster scaffold homolog (E. coli)	*ISCU*	-1.60	0.0157
CK948574	beta-2-microglobulin	*B2M*	-1.60	0.0189
CB426311	UDP-glucose ceramide glucosyltransferase	*UGCG*	-1.59	0.0498
CB168007	reticulocalbin 1, EF-hand calcium binding domain	*RCN1*	-1.59	0.0012

CK959644	MYC induced nuclear antigen	*MINA*	-1.59	0.0012
CK972308	UBX domain protein 4	*UBXN4*	-1.59	0.0418
CK846207	synaptogyrin 1	*SYNGR1*	-1.59	0.0001
CK976524	metadherin	*MTDH*	-1.59	0.0349
CB530355	similar to Nckap1 protein	*LOC510404*	-1.59	0.0098
CK771637	flotillin 1	*FLOT1*	-1.59	0.0003
CK849625	cathepsin H	*CTSH*	-1.59	0.0011
BP102272	X (inactive)-specific transcript	*XIST*	1.50	0.0479
CK726980	UMC-bemiv_0B02-002-d05 Metaphase II stage oocyte bemiv Bos taurus cDNA 3-, mRNA sequence	---	1.52	0.0059
CK943621	4067664 BARC 10BOV Bos taurus cDNA clone 10BOV15_E01 3-, mRNA sequence	---	1.55	0.0118
CK948835	sorbin and SH3 domain-containing protein 1	*SORBS1*	1.56	0.0113
BE665099	forkhead box P4	*FOXP4*	1.60	0.0369
CK774445	similar to Transforming growth factor-beta induced protein IG-H3 precursor (Beta IG-H3) (Kerato-epithelin) (RGD-containing collagen associated protein) (RGD-CAP)	*LOC539596*	1.61	0.0144
BE667577	CKLF-like MARVEL transmembrane domain containing 7	*CMTM7*	1.61	0.0111
CK957215	4097701 BARC 10BOV Bos taurus cDNA clone 10BOV3_P12 3-, mRNA sequence	---	1.62	0.0165
CB424435	nicotinamide nucleotide adenylyltransferase 1	*NMNAT1*	1.66	0.0336
CK776579	similar to NEPH1 protein	*LOC781667*	1.71	0.0004
BF776493	similar to WNK lysine deficient protein kinase 4	---	1.78	0.0155
CB441821	similar to mCG142710	*LOC511229*	1.84	0.0035
CB461078	721004 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	1.93	0.0109
NM_174844.1	glycine cleavage system protein H (aminomethyl carrier)	*GCSH*	1.99	0.0292
AW632179	potassium channel tetramerisation domain containing 1	*KCTD1*	2.92	0.0036
CB535261	768672 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	3.08	0.0007

Supplementary Table 4.
List of differentially expressed transcripts regarding the interaction between subspecies (*B. taurus indicus* – Nellore vs. *B. taurus taurus* - Simmental) and origin (ET vs IVP).

Representative Public ID	Gene Title	Gene Symbol	Fold change	*P*-value
BP108263	secreted seminal-vesicle Ly-6 protein 1	*SSLP1*	1.67	0.0158
CK955677	keratin 19	*KRT19*	1.57	0.0145
CB166446	nucleobindin 2	*NUCB2*	1.55	0.0353
CK776789	similar to trophoblast Kunitz domain protein 5	*LOC618696*	1.54	0.0449
CK958396	destrin (actin depolymerizing factor)	*DSTN*	-1.59	0.0033
CB424435	nicotinamide nucleotide adenylyltransferase 1	*NMNAT1*	-1.60	0.0381
CK942763	similar to Tropomyosin alpha-1 chain (Tropomyosin-1) (Alpha-tropomyosin) /// tropomyosin 1 (alpha)	*LOC788816 /// TPM1*	-1.60	0.0149
CB172313	ribosomal protein L7-like 1	*RPL7L1*	-1.61	0.0197
CK848123	970838 BARC 5BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.61	0.0105
CK775514	squamous cell carcinoma antigen recognized by T cells 3	*SART3*	-1.61	0.0241
CB171498	ribosomal protein L15	*RPL15*	-1.62	0.0041
CK958448	tropomyosin 3	*TPM3*	-1.63	0.0250
CK849123	eukaryotic translation initiation factor 3, subunit G	*EIF3G*	-1.64	0.0473
BE749511	tripartite motif-containing 2	*TRIM2*	-1.64	0.0472
NM_175795.2	cytochrome c oxidase subunit VIIb	*COX7B*	-1.65	0.0242
CB447648	Carnitine palmitoyltransferase 1A (liver)	*CPT1A*	-1.65	0.0362
CB461923	SET domain and mariner transposase fusion gene	*SETMAR*	-1.66	0.0423
AU276758	phosphodiesterase 4D interacting protein	*PDE4DIP*	-1.66	0.0498
CK775725	BMS1 homolog, ribosome assembly protein (yeast)	*BMS1*	-1.67	0.0113
NM_174313.2	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	*FABP3*	-1.68	0.0249
BP109396	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	*SLC25A20*	-1.68	0.0147
BI537914	428203 MARC 4BOV Bos taurus cDNA 5-, mRNA sequence	---	-1.68	0.0156

BP102272	X (inactive)-specific transcript	*XIST*	-1.69	0.0136
AV594069	ribosomal protein L36	*RPL36*	-1.70	0.0339
CK847594	hypothetical LOC506494	*MGC159500*	-1.70	0.0460
CK776519	967837 MARC 4BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.71	0.0342
CB531801	signal sequence receptor, delta (translocon-associated protein delta)	*SSR4*	-1.72	0.0023
NM_176614.1	pregnancy-associated glycoprotein 2	*PAG2*	-1.73	0.0047
BM445534	PREDICTED: Bos taurus uncharacterized LOC100849050 (LOC100849050), miscRNA	---	-1.75	0.0040
NM_174844.1	glycine cleavage system protein H (aminomethyl carrier)	*GCSH*	-1.81	0.0337
CB461078	721004 MARC 6BOV Bos taurus cDNA 3-, mRNA sequence	---	-1.82	0.0095
CK778646	proteasome (prosome, macropain) assembly chaperone 1	*PSMG1*	-1.83	0.0491
AU275257	ribosomal protein L18	*RPL18*	-1.85	0.0380
CK948858	selenophosphate synthetase 2	*SEPHS2*	-1.85	0.0272
CK777978	phosphoribosyl pyrophosphate synthetase 1	*PRPS1*	-1.86	0.0010
BE749963	alanyl-tRNA synthetase	*AARS*	-1.90	0.0332
AU233180	Tropomyosin 1 (alpha)	*TPM1*	-1.91	0.0164
NM_174763.2	peroxiredoxin 2	*PRDX2*	-1.92	0.0292
BM435101	zinc finger protein 36, C3H type-like 1	*ZFP36L1*	-1.94	0.0394
NM_174334.2	hydroxysteroid (17-beta) dehydrogenase 10	*HSD17B10*	-1.97	0.0124
CK963170	ribosomal protein L7a	*RPL7A*	-2.03	0.0098
CK971789	ubiquitin-conjugating enzyme E2 variant 1	*UBE2V1*	-2.16	0.0189
CB464010	mitochondrial carrier homolog 1 (C. elegans)	*MTCH1*	-2.16	0.0369
CK963094	hypothetical protein MGC127538	*MGC127538*	-2.17	0.0196
CB464808	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	*PRKAR1A*	-2.17	0.0313
CK728409	cornichon homolog 4 (Drosophila)	*CNIH4*	-2.17	0.0416
CK968451	glycyl-tRNA synthetase	*GARS*	-2.21	0.0338
AY192438.1	thymosin beta 4, X-linked	*TMSB4X*	-2.33	0.0326
CK780160	ribosomal protein S27-like	*RPS27L*	-2.39	0.0483

AW485507	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	*EIF2S3*	-2.40	0.0300
CK772180	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	*ATP6V0D2*	-2.42	0.0029
CK846999	glutamyl aminopeptidase (aminopeptidase A)	*ENPEP*	-2.49	0.0175
CK953606	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	*CYP3A28*	-2.51	0.0483

4- CONCLUSÕES

1- Os blastocistos Simental apresentaram maior sobrevivência após a vitrificação (34,6 vs 20,2%) e conteúdo lipídico (3,4±0,3 vs 2,4±0,3 gray intensity x 10^{-4} / μm^3) do que os Nelore, respectivamente.

2- Os blastocistos TE apresentaram maior sobrevivência após a vitrificação (38,5 vs 18,1%) e menor conteúdo lipídico (2.1±0.3 vs 4.0±0.3 gray intensity x 10^{-4} / μm^3) em relação aos PIV, respectivamente.

3- O perfil dos lipídeos de membrana do tipo fosfocolinas (PC) e esfingomielinas (SM) foi característico para cada grupo; sugerindo marcadores positivos [PC (34:2) e PC (36:5)] e negativos [PC (32:0) e PC (34:1)] da criotolerância embrionária.

4- O padrão de expressão gênica global foi característico para cada grupo experimental.

5- Foi possível relacionar a composição lipídica e o padrão de expressão gênica global com a sobrevivência embrionária após a criopreservação.

6- Genes relacionados ao metabolismo lipídico *AUH* e *ELOVL6*, *ACSL3* e *ACSL6*, estavam *upregulated,* respectivamente nos embriões Simental e PIV, podendo estar associados aos perfis lipídico distintos e a capacidade de sobrevivência embrionária após a criopreservação.

7- Genes relacionados ao metabolismo mitocondrial (*ATP5B*), estresse oxidativo (*GPX4*), anti- (*DAD1*) e pró- (*DAP*) apoptose, estresse térmico (*HSPA5*), prenhez (PAG2 e IFNT2) e diferenciação (*KRT18*) podem estar associados a qualidade e capacidade de sobrevivência embrionária após a criopreservação.

8- Os dados gerados neste estudo forneceram diferentes assinaturas do trancriptoma de um modelo utilizando duas subespécies bovinas e duas origens embrionárias com conteúdo lipídico, perfil lipídico e capacidade de sobrevivência embrionária após a criopreservação específicos.

5- CONSIDERAÇÕES FINAIS

A partir do uso de blastocistos oriundos de duas subespécies (*Bos taurus taurus e Bos taurus indicus*) e duas origens (PIV e TE), foi possível estabelecer um modelo experimental com alto e baixo conteúdo lipídico e criotolerância embrionária, sem mimetizar esta condição in vitro com o uso de aditivos e produtos químicos.

Enquanto que no efeito de origem (PIV vs TE) foi observado que o elevado conteúdo lipídico dos embriões PIV afetou sua criotolerância embrionária comparado aos TE, concordando com a literatura. Quando foi incluído no modelo experimental o efeito de subespécies, o elevado conteúdo lipídico observado nos blastocistos Sementais não reduziu sua criotolerância comparado aos Nelores, sustentando a hipótese de que a capacidade do embrião sobreviver após a criopreservação é um evento multifatorial, e não dependente apenas do conteúdo lipídico.

Aparentemente o conteúdo lipídico é uma consequência de uma série de eventos depreciativos da qualidade dos embriões PIV ao longo do cultivo *in vitro*, destacando-se a provável teoria do “unquiet metabolism”, ou seja, a ocorrência de um metabolismo exacerbado o que foi evidenciado pelo grande número de genes diferentemente expressos entre os embriões PIV e TE.

Foi constatado que o perfil lipídico da membrana celular e o padrão de expressão gênica global afetam a criotolerância embrionária. Além disso, foi possível associar genes diferentemente expressos entre os diversos grupos experimentais potencialmente envolvidos no metabolismo dos fosfolipídeos de membrana que abriram a possibilidade do estudo da modulação enzimática envolvida em tal evento.

Um grande número de dados foi gerado a partir das análises de lipidômica e transcriptômica que forneceu informações valiosas para condução de uma análise holística pela abordagem da Biologia de Sistemas com o objetivo de estabelecer assinaturas moleculares envolvidas no metabolismo lipídico, viabilidade embrionária e capacidade de sobrevivência após a criopreservação. Além disso, tais informações servirão de suporte para condução de experimentos funcionais futuros, envolvendo as moléculas identificadas, para desvendar os mecanismos envolvidos na criotolerância embrionária.

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