

# Lipid structural information from a single equine embryo by MALDI-MS

## *Informação estrutural de lipídios a partir de um único embrião equino pela técnica de MALDI-MS*

Roseli Fernandes GONÇALVES<sup>1</sup>; Christina Ramires FERREIRA<sup>2,3</sup>; Cássia Maria Barroso ORLANDI<sup>4</sup>; Sérgio Adriano SARAIVA<sup>3</sup>; Heder Nunes FERREIRA<sup>5</sup>; Eduardo Jorge PILAU<sup>6</sup>; Fabio César GOZZO<sup>6</sup>; Marcos Antônio de ACHILLES<sup>7,8</sup>; Daniela Lazzaro D'ERCOLE<sup>8</sup>; Marcos Nogueira EBERLIN<sup>3</sup>; José Antônio VISINTIN<sup>1</sup>

<sup>1</sup> Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Reprodução Animal, São Paulo – SP, Brazil

<sup>2</sup> Aston Laboratory for Mass Spectrometry Laboratory, Chemistry Department, Purdue University, West Lafayette – IN, United States of America

<sup>3</sup> Universidade de Campinas, Instituto de Química, ThoMson Mass Spectrometry Laboratory, Campinas – SP, Brazil

<sup>4</sup> Universidade Estadual Paulista, Faculdade de Medicina Veterinária, Laboratório de Endocrinologia Animal, Araçatuba – SP, Brazil

<sup>5</sup> Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Reprodução Animal e Radiologia Veterinária, Botucatu – SP, Brazil

<sup>6</sup> Universidade de Campinas, Instituto de Química, Dalton Mass Spectrometry Laboratory, Campinas – SP, Brazil

<sup>7</sup> Instituto Biológico, Centro de Pesquisa e Desenvolvimento de Sanidade Animal, São Paulo – SP, Brazil

<sup>8</sup> Achilles Genetics, Garça – SP, Brazil

### Abstract

It was reported the potential of MALDI-MS for the characterization of lipid species present in a single equine embryo, and studied some lipid structures detected by collision induced dissociation (CID) experiments. In the positive ion mode spectrum, it were observed mostly protonated and sodiated species of sphingomyelins (SM), phosphatidylcholines (PC) and triacylglycerols (TAG). In the negative ion mode, it were observed phosphatidylethanolamines (PE) and phosphatidylinositols (PI). MS/MS spectrum of most intense lipid ions was performed to show MALDI-MS/MS structural information potential. MS/MS spectrum in the positive mode of  $m/z$  760.6 (attributed as PC34:1) depicted characteristic PC fragments of  $m/z$  184.1 (choline polar head), and the neutral loss (NL) of 183 (phosphorylcholine). For the ion of  $m/z$  766.6 (attributed as PE 38:5), we observed the NL of 140, characteristic of PE. For the ion of  $m/z$  808.7 (attributed as PC 38:5), besides the fragment at  $m/z$  184.1 at the NL of 183, it was possible to observe the loss of trimethylamine (ion of  $m/z$  749.6), and the cyclophosphane (ion of  $m/z$  147.0). Finally, for the negative ion mode, we isolated and fragmented the ion at  $m/z$  863.6, which was attributed as PI 36:1 due to the presence of  $m/z$  153 (glycerol phosphate –  $H_2O-H$ ), 223 (phospho inositol –  $2H_2O-H$ ), 241 (phospho inositol –  $H_2O-H$ ), 281 (oleic acid), and 581.3 (lysophosphoinositol –  $H_2O-H$ ). It was concluded that MALDI-MS allowed the detection of a broad range of PC, SM, PE, PI and TAG lipid species, as well as a fast and confident characterization of lipid structures from a single equine embryo.

**Keywords:** Equine. Embryo. Lipid species. MALDI-MS.

### Resumo

É relatado o potencial da técnica de MALDI-MS para caracterizar espécies de lipídios presentes em um único embrião equino e estudadas algumas estruturas lipídicas detectadas por dissociação induzida por colisão (CID). No espectro de modo íon positivo, foram observadas espécies, principalmente, protonadas e sodiadas de esfingomielinas (SM), fosfatidileolinas (PC) e triacilgliceróis (TAG). No modo negativo, foram observadas fosfatidiletanolaminas (PE) e fosfatidilinositos (PI). Espectros de íons de lipídios com maior intensidade foram utilizados para demonstrar o potencial da informação estrutural por MALDI-MS/MS. O espectro no modo positivo de  $m/z$  (massa sobre carga) 760,6 (atribuída como PC34:1) apresentou características de fragmentos PC de  $m/z$  184,1 (denominada cabeça polar de colina), além de perda neutral (NL) de  $m/z$  183 (fosforilcolina). Para o íon de  $m/z$  766,6 (atribuída como PE38:5), observou-se a NL de 140, característica do PE. Para o íon de  $m/z$  808,7 (38,5 atribuído como PC), além do fragmento  $m/z$  184,1 na NL de 183, foi possível observar a perda de trimetilamina (íon de  $m/z$  749,6) e o ciclofosfano (íon de  $m/z$  147,0). Finalmente, para o modo de íon negativo, foram isolados e fragmentados o íon de  $m/z$  863,6 que foi atribuído como PI36:1, devido à presença de  $m/z$  153 (fosfato de glicerol –  $H_2O-H$ ), 223 (inositol fosfo –  $2H_2O-H$ ), 241 (fosfoinositol –  $H_2O-H$ ), 281 (ácido oleico) e 581,3 (lisofosfoinositol –  $H_2O+H$ ). Foi concluído que a MALDI - MS permite a detecção de uma ampla gama de espécies de PC, SM, PE, PI e TAG lipídicas, bem como a caracterização rápida e confiante de estruturas lipídicas a partir de um único embrião equino.

**Palavras-chave:** Equino. Embrião. Perfil lipídico. MALDI-MS.

\*Roseli F. Gonçalves, Christina R. Ferreira and Cassia M. B. Orlandi contributed equally to this work.

**Correspondence to:**

Roseli Fernandes Gonçalves  
 Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia  
 do Departamento de Reprodução Animal  
 Av. Prof. Dr. Orlando Marques de Paiva, 87 – Cidade Universitária  
 CEP 05508-270, São Paulo, SP, Brazil  
 e-mail: rfgoncalves@gmail.com

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## Introduction

The use of embryo transfer and the development of assisted reproductive techniques for the horse industry have increased steadily over the past two decades. However, techniques that involve the manipulation of oocytes and/or embryos may themselves be detrimental to embryo viability and subsequent development (ALLEN, 2005; COUTINHO DA SILVA, 2008; SQUIRES et al., 2003). Quality of the equine embryo has a major effect on pregnancy rates, and embryo cryopreservation (ALLEN, 2005; LAGARES et al., 2009; SQUIRES, 2005).

Lipid species in the cell mostly reside in the membranes, forming the lipid bilayer, which supports an array of proteins. Lipids are not just “building blocks” in cells; they also play active roles in various cellular functions and can form 9,000 to 100,000 different molecular species (BENNETT; TIELEMAN, 2013). Numerous studies have indicated that the lipid composition of an oocyte or embryo can affect its viability after cryopreservation (APPARICIO et al., 2012; SUDANO et al., 2012). Lipid composition may also be related to embryo viability, and omics-based technologies should contribute significantly with the comprehension of this question (FERREIRA et al., 2010, 2012a, b, 2014; GONÇALVES et al., 2014).

Therefore, an objective method of assessing viability of embryos before and/or after oocyte/embryo manipulation is desirable. At this time, morphologic evaluation is the most widely used method for determination of the equine embryo viability. Although morphologic assessment of embryo quality will not always accurately predict the survival of

individual embryos, it is very useful for establishing the survival of groups of embryos (ROYÈRE et al., 2009). Recently, global assessment strategies involving genomic, transcriptomic, proteomic, or metabolomic profiling of oocytes, granulosa or cumulus cells, and embryos have been applied to assisted reproduction (ASSOU et al., 2010; ROYÈRE et al., 2009).

In a previous study, it was reported a direct analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) of single and intact embryos or oocytes from different species (APPARICIO et al., 2012; FERREIRA et al., 2010, 2012a, b). MALDI-MS allows not only a fast and efficient detection of various lipids classes, but also the study of their structure by fragmentation (MS/MS) experiments. The aim of this study was to characterize the lipid classes present in a single equine embryo (day 8 and 9) by MALDI-MS, and to study some lipid structures by collision induced dissociation (CID) experiments.

## Materials and Methods

All chemicals in this study were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated.

### Animals

The care and use of equine samples was approved by the Institutional Committee for Ethics in Animal Research at the University of Campinas (UNICAMP). The institution follows the Ethical Principles of Animal Research established by the Brazilian College for Animal Experimentation (COBEA), filed under protocol number 2819-1.

### Embryo recovery

An eight-year old, quarter horse mare was maintained at the paddock in a private Veterinary Teaching Hospital – Reproductive Unit (Northeast – Paraná state) being used as an embryo donor mare at the reproductive season; during summer. The mare received daily free choice hay, concentrate,

mineral salt and water. After rectal palpation followed by ultrasound evaluation (7.5 MHz transrectal probe attached to a Honda HS-2000VET), the mare was determined to be cycling normally and ovulation was induced (hCG 2500 iu, Vetecor®, Hertape Calier, Brazil) upon detection of a 35mm follicle and presence of uterine edema. Fresh semen ( $500 \times 10^6$  viable sperm) was used for AI, approximately within 24 hours after ovulation being induced and embryo was recovered using Lactate Ringer solution (Nutricell®, Campinas, SP, Brazil), eight days after ovulation was detected. The embryo was placed in a Holding Plus Medium with 0.4 % BSA (Vitrocell®, Campinas, SP, Brazil), refrigerated and transported at 15°C to the ThoMSon Laboratory within 72 hours. At the subsequent cycle, the same mare was followed by reproductive examination and ovulation was induced as described above. The same procedure for AI was used and another embryo was recovered at nine days after ovulation. The embryo storage and transportation was performed in the same condition as described before. Total of two cycles from the same mare were used to obtain two embryos that were further processed for lipid content evaluation at the referred laboratory.

#### **Lipid analysis by MALDI-MS**

The embryos collected as described above were washed twice with PBS (Nutricell®) and stored in microtubes containing 100  $\mu$ L of a 50% aqueous methanol solution at -80°C until analysis. Sample preparation involved placing each embryo in a given spot of the MALDI target plate (Waters, Manchester, UK) under the stereomicroscope. Samples were allowed to dry at room temperature and their location was recorded in order to place the laser at the correct location during analysis. Just before analysis, 1  $\mu$ L of 1.0 mol/L 2,5-dihydroxybenzoic acid (DHB) in methanol was placed in each target spot and allowed to dry at room temperature.

MALDI-MS spectra were acquired in the positive (using 2,5-dihydroxybenzoic acid as MALDI matrix) and negative (using -aminoacridine hemihydrate

as MALDI matrix) ion modes in a Q-ToF Premier (Synapt HDMS) mass spectrometer (Waters) equipped with a 200 Hz solid-state laser in the  $m/z$  400-1000 range, operated in the reflectron and QTOF modes. Therefore, unmodified (no extraction procedure) embryos have been directly analyzed. Spectra processing was performed using the MassLynx 4.0 software (Waters Corp., Milford, MA, USA). Besides obtaining the lipid profile at both ion modes, some ions were isolated in the positive and in the negative ion mode for MS/MS experiments. Figure 1 illustrates the sample preparation procedure and information on lipids present in the MALDI mass spectra.

#### **Data analysis**

The MALDI mass spectra of each sample were accumulated using MarkerLynx 4.0 software (Waters, Manchester, UK) and exported for principal component analysis (PCA by MarkerLynx™ XS, Waters, Manchester, UK). The following were the methodology parameters: mass tolerance = 0.5 Da, baseline noise = 50 and intensity threshold (count) = 1000 with deisotope MS data.

#### **Results**

Besides obtaining the lipid profile at both ion modes, some ions were isolated in the positive and in the negative ion mode for MS/MS experiments. The lipid profile of positive ion mode was mainly represented by phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingomyelins (SM), and triacylglycerols (TAG) species, while in the negative ion mode, PE and phosphatidylinositols (PI) were detected (Figure 2). MS/MS spectrum in the positive mode of  $m/z$  760.6 (attributed as PC 34:1) depicted characteristic PC fragments of  $m/z$  184.1 (choline polar head), and the neutral loss (NL) of 183 (phosphorylcholine). For the ion of  $m/z$  766.6 (attributed as PE 38:5), we observed the NL of 140, characteristic of PE. For the ion of  $m/z$  808.7 (attributed as PC 38:5), besides the fragment

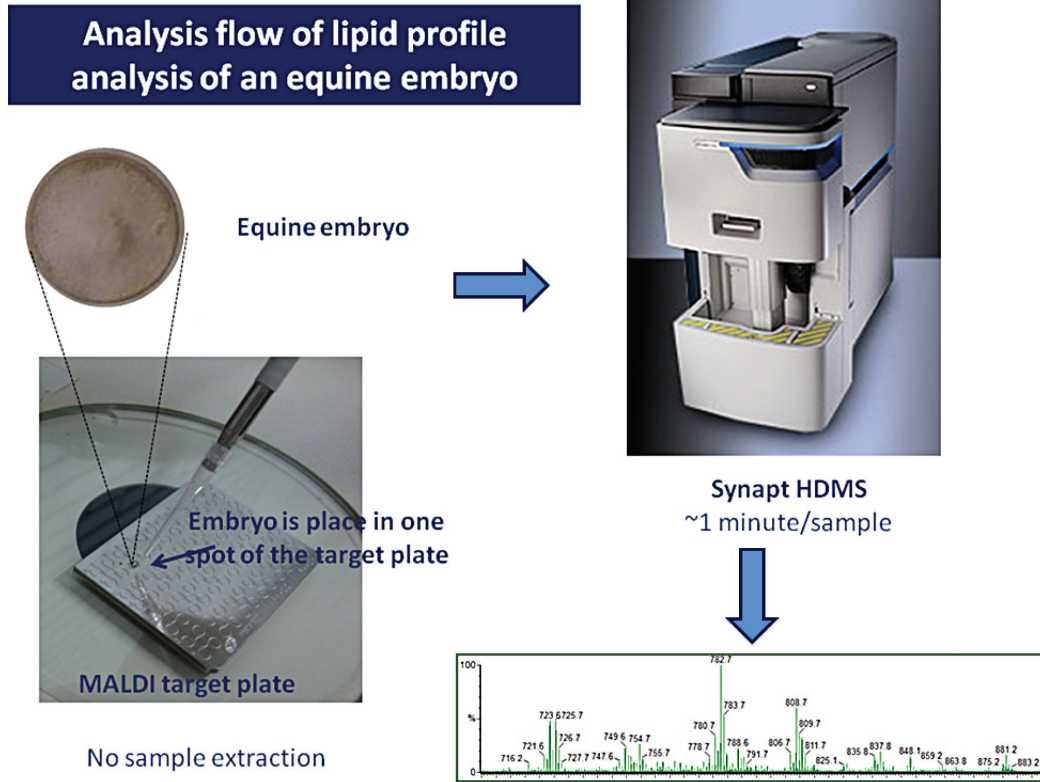


Figure 1 – Schematic of sample preparation and analysis by MALDI-TOF MS with DHB matrix of a single embryo horse  
 Source: (FERREIRA, 2014)

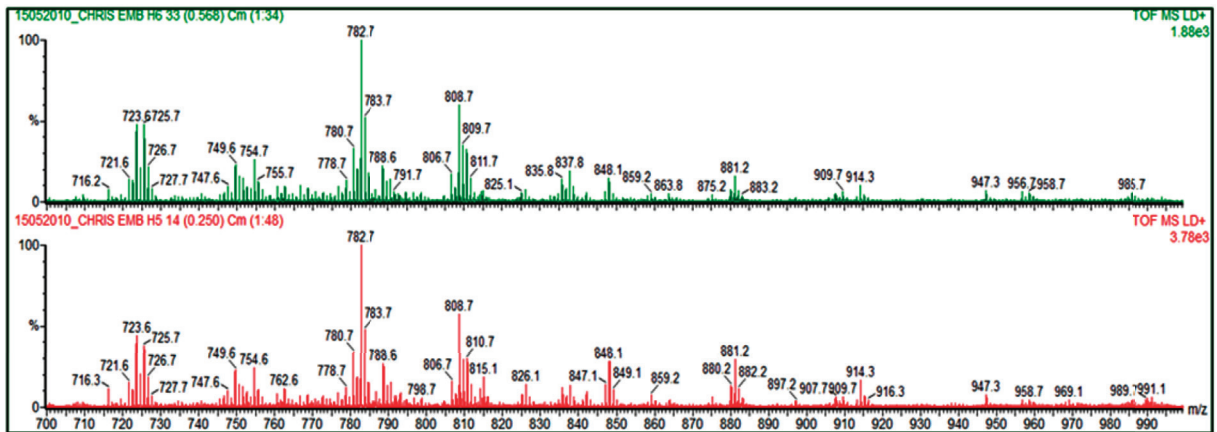


Figure 2 – Mass spectra tags indicating an equine embryo. The spectra were obtained by MALDI-TOF MS with DHB matrix  
 Source: (FERREIRA, 2014)

at  $m/z$  184.1 at the NL of 183, it was observed the loss of trimethylamine (ion at  $m/z$  749.6), and the cyclophosphane (ion at  $m/z$  147.0). Finally, for the negative ion mode, we isolated and fragmented the ion at  $m/z$  863.6, which was attributed as PI 36:1 due

to the presence of various characteristic ions reported for this lipid species: At  $m/z$  153 (glycerol phosphate -H<sub>2</sub>O-H), 223 (phospho inositol-2H<sub>2</sub>O-H), 241 (phospho inositol-H<sub>2</sub>O-H), 281 (oleic acid), and 581.3 (lysophosphoinositol-H<sub>2</sub>O-H).

## Discussion

Lipid content of oocytes and embryos is an important parameter linked to both quality and cryotolerance. Physical changes of lipids submitted to freezing temperatures are among the major causes of cellular cryodamage. Not only intracellular lipids, but also the lipid composition of the membranes contributes to differences in sensitivity to cooling injuries. Some factors, such as the origin of the oocyte or the embryo (*in vitro* produced or *in vivo* derived, species, breed, physiological state and nutrition of donor), proved to be determinant for the lipid content (ABE et al., 2002; GENICOT et al., 2005; McEVOY et al., 2000; STEEL; HASLER, 2004; ZERON et al., 2002).

Most information regarding lipid structural analysis from oocytes and embryos has been obtained by staining methods, such as Nile Red, which quantify cytoplasmic lipids in general and gas chromatography (GC) that quantify by fatty acid (FA) residues extracted from the total sample lipid content (BARCELÓ-FIMBRES; SEIDEL JR., 2011; KIM et al., 2001; LAPA et al., 2011; MATORRAS et al., 1998).

Actually, MALDI-MS has been used for the direct and concomitant assess of the profiles and chemical structures of both intact phospholipids and triacylglycerols present in single oocytes and embryos. Ferreira et al. (2010) were the first to report the lipid profile study of individual mammal embryo and oocyte, without the need of pooling from dozens to hundreds. The data collection is rapid, highly sensitive, can tolerate some level of impurities, and easy to interpret.

The MALDI-MS allowed the detection of PC, SM, PE, PI and TAG lipid species, as well as fast and

confident characterization of its lipid structure from a single equine embryo. These results may contribute to a better knowledge of equine embryo metabolism and to cryopreservation studies. Genomic, transcriptomic, proteomic, and metabolomic analysis strategies (the omics) are being rapidly applied to the field of oocyte and embryo assessment, leading to discovery of biomarkers associated with oocyte and embryo viability. In addition, the clinical predictive value of some of these approaches has been demonstrated in blind trials. While randomized prospective trials assessing implantation and pregnancy rates using these technologies (used alone or in combination with morphology), compared to conventional morphologic assessment are lacking, recent reports are quite encouraging. Therefore, a fast, inexpensive, easy-to-use, and hopefully non-invasive test involving one or a combination of these parameters may soon be available in clinical practice.

In conclusion, MALDI-MS with minimal sample preparation demonstrated to be a rapid and efficient technique for screening lipid profile of equine embryos. To our knowledge, this was the first study addressing lipid profile in equine embryos, but the analysis of a greater number of embryos as well as different development periods will contribute to building a database of lipid profiles that allows a better understanding of the lipid physiology.

### ***Declaration of interest and Acknowledgements***

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The authors thank FAPESP (São Paulo Research Foundation; 2010/01077-9 and 2011/18085-7).

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