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Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach

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ABSTRACT

Characterisation of the physiologic equine amniotic fluid (AF) proteome is a prerequisite to study its changes during diseases and discover new biomarkers. The aim of this study was to identify by a proteomic approach the most abundant proteins of equine AF. AF samples were collected at parturition from 24 healthy mares that delivered healthy foals. All samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% gels. A pool of the 24 samples, after SDS-PAGE, was cut in 25 slices, trypsin-digested and analysed by mass spectrometry (MS) for protein identification. Mean AF protein concentration was 1.96 ± 1.12 g/L. Thirty-four proteins were successfully identified by MS and subsequently categorised according to Gene Ontology (GO). Twelve proteins (e.g. fibronectin, lumican, thrombospondin and fibulin) belonged to or interacted with the extracellular matrix (ECM) playing an important role in the development of foetal tissues. Most of the remaining proteins were classified as transport (e.g. albumin, major allergen Equ c1 and alpha-fetoprotein) delivering nutrients, ions and lipids essential for foetal growth and development. Among these proteins, major allergen Equ c1 is widely studied in human medicine because it induces Ig-E mediated type I allergic reaction. The absence of immunoglobulins in equine AF was also confirmed.

1. Introduction

Amniotic fluid (AF) is a complex dynamic milieu that changes as pregnancy progresses. AF contains nutrients and growth factors that facilitate foetal growth, provides mechanical cushioning and antimicrobial effectors to protect the foetus and allows assessment of foetal maturity and disease (Underwood et al., 2005). In comparison to humans, the physiology and pathophysiology of foetal fluids in domestic mammals are poorly understood (Canisso et al., 2015). In horses, some studies have investigated the biochemical composition, particularly enzymes and electrolytes, in AF collected by ultrasound-guided transabdominal amniocentesis, at delivery or at slaughter (Holdstock et al., 1995; Lyle et al., 2006; Williams et al., 1993; Zanella et al., 2014). AF was also studied for evaluation of foal's lung maturity at birth through lecithin/sphingomyelin ratio and lamellar body count (Castagnetti et al., 2007). More recently, significantly higher levels of lactate were found in AF collected during parturition in mares delivering healthy foals (Pirrone et al., 2012).

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Unlike the allantoic fluid, equine AF can be easily collected during parturition without stressing the animal and avoiding any contamination (Castagnetti et al., 2007; Pirrone et al., 2012). As reported in women, the biochemical composition of AF, including proteins, is primarily representative of the foetal profile and reflects its physiological status (Tong, 2013), thus it could be potentially useful to evaluate the high-risk foal born attended.

Proteomics is a powerful analytical approach providing a profile of proteins present in a biological sample at a given time. The high potential of this approach has been recently found to have a major role in different areas of veterinary medicine, from farm (Almeida et al., 2015) to companion (Ferlizza et al., 2015; Miller et al., 2014) animals. Proteomic techniques have recently been applied to the characterisation of horse amniotic membrane (Galera et al., 2015) and bovine conceptus fluids (Riding et al., 2008), whereas the equine AF proteome remains uncharacterised. Therefore, the aim of this study was to identify the most abundant proteins in equine AF by SDS-PAGE separation followed by mass spectrometry identification.

2. Materials and methods

2.1. Animal selection and data collection

Twenty-four mares admitted for assisted delivery during three breeding seasons at the S. Belluzzi Equine Perinatology Unit of the Department of Veterinary Medical Sciences, University of Bologna, were included. The mares were hospitalised at about 310 days of pregnancy because the owners requested an attended parturition, and remained under observation for at least 7 days *postpartum*. They were housed in wide straw bedding boxes and fed with hay *ad libitum* and concentrates twice a day. During the day, the mares were allowed to go to pasture.

All the mares included in the study were healthy based on clinical and ultrasonographic evaluation. At admission, a complete clinical evaluation, including complete blood count, serum biochemistry and transrectal ultrasonography, was performed. Severe maternal illness, uterine discharge, premature lactation, twinning, abnormal foetal presentation, placenta oedema and signs of foetal distress were ruled out. During the course of hospitalisation, mares were clinically evaluated twice a day and by transrectal ultrasonography every 10 days until parturition. The following ultrasonographic parameters were evaluated: combined thickness of the uterus and placenta, foetal fluids echogenicity, foetal activity, and foetal orbital area. Foals were born between 320 and 365 days of pregnancy by normal delivery, had an Apgar score $\geq$8 recorded within 5 min from birth (Vaala et al., 2002) and had a normal clinical evaluation during the course of hospitalisation, including a complete blood count and serum biochemistry at birth and an immunoglobulin G (IgG) serum concentration $\geq$800 mg/dL at 18–24 h of life.

For each mare, the following data were recorded: breed, age, parity, days of pregnancy, body weight, length of stage II labour (minutes), and foal's body weight and Apgar score. All procedures on the animals were carried out with the approval of the Ethical Committee, in accordance with DL 116/92, approved by the Ministry of Health (approval number: n.18/64/11; date of approval 22/02/2011). Oral informed consent was given by the owners.

2.2. Sample collection

At foaling, a sample of AF was collected from each mare with a 50 mL syringe by needle puncture of the amniotic sac within few minutes of its appearance through the vulva during stage II of labour. The AF was then immediately transferred to 5 mL test tubes and stored at −80 °C until SDS-PAGE and protein identification were performed. AF protein concentration was determined by the Biuret method using bovine serum albumin as standard.

2.3. SDS-PAGE

To optimise protein separation, different protocols were tested including 4–12% and 12% polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 2-(*N*-morpholino) propanesulfonic acid buffer (MOPS; NuPage/Thermo Fisher Scientific) or 2-(*N*-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific) with sodium dodecyl sulphate (SDS). Each AF sample (n = 24) was analysed at least twice with the protocol assuring the best protein separation in our experimental conditions (4–12% gels, in MOPS buffer). Twenty μg of proteins were loaded for each sample and the gels were stained with Coomassie G250 compatible with mass spectrometry analysis. After staining, each gel was digitalised by ChemiDoc^{TM}MP (BioRad, Hercules, California, USA) and pherograms were obtained using ImageLab 5.2.1 software (BioRad). The software determines the volume of each protein band through the analysis of the pixel values in the digital image, meaning as volume the sum of all the pixels intensities within the band boundaries. The band volumes are subsequently compared to the entire volume of the lane and the relative abundances reported in percentage. A pool was prepared by collecting and mixing 50 μg of protein from each AF (n = 24) and analysed twice with the same protocol used for each sample.

2.4. Protein identification by mass spectrometry

The pool lanes were divided manually into 25 slices and subjected to in-gel tryptic digestion as previously described (Bellei et al., 2013). Digested dried samples were then resuspended in 97% water/3% ACN added of 1% formic acid (Buffer A) and analysed by a Nano LC-CHIP-MS system (ESI-Q-TOF 6520; Agilent Technologies, Santa Clara, California, USA). Four microliters of each sample were loaded into the system and transported to the Chip enrichment column (Zorbax C18, 4 mm × 5 μm i.d., Agilent Technologies) by a capillary pump, with a loading flow of 4 μL/min, using 95% ACN/5%water added of 0.1% formic acid (buffer B) as mobile phase. Nitrogen was used as the nebulising gas. A separation column (Zorbax C18, 43 mm × 75 μm i.d., Agilent Technologies), at flow rate of 0.4 μL, was used for peptide separation.

Since the horse protein database is not well annotated, a broader taxonomy, namely "all mammals", was selected for