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Lipidomics of equine amniotic fluid: Identification of amphiphilic (O-acyl)-ω-hydroxy-fatty acids

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ABSTRACT

Amniotic fluid is essential for the growth and maturation of the fetus prior to parturition. While our knowledge of human amniotic fluid is extensive, current data for equine amniotic fluid is limited. We therefore undertook a detailed lipidomics analysis of equine amniotic fluid. Using a non-targeted high-resolution mass spectrometric lipidomics analysis of equine amniotic fluid, we were able to characterize a diverse array of individual lipids. This non-biased analytical approach detected, for the first time, the presence of (O-acyl)-ω-hydroxy-fatty acids (OAHFA) with up to 52 carbon chain lengths in amniotic fluid. The identities of these lipid amphiphiles were validated both by high-resolution mass spectrometry and by tandem mass spectrometry (<2 ppm mass error) which identified the fatty acid and hydroxy-fatty acid components of individual OAHFAs. The only previous identification of OAHFAs has been in sperm and meibomian glands, and their sebaceous secretions, suggesting that these lipids may have unique functional roles in highly specialized compartments. The amphiphilic and surfactant properties of these unique lipids could provide an interface between amniotic lipids and fetal skin and/or lungs. The potential roles of OAHFAs as well as their source in amniotic fluid remain to be explored based upon these novel lipidomics findings but our study of the developmental time course of amniotic OAHFAs suggest that they may act as lubricants in delivery and/or a role in the development of fetal lung function around parturition.

1. Introduction

Amniotic fluid is a complex biofluid which provides both mechanical and antimicrobial protection to the fetus as well as lubrication, nutrition, and growth factors which are important in fetal development [1]. Amniotic fluid is formed with contributions from urine, oral, nasopharyngeal, tracheal and pulmonary secretions as well as transfer across the amniotic membrane (reviewed by [2]). A number of studies have characterized the biochemical composition of equine amniotic fluid during various stages of gestation [3–7]. To date, studies that have addressed the lipid composition of amniotic fluid in the horse have examined the lecithin:sphingomyelin (L/S) ratio in amniotic fluid which has been used as a measure of equine fetal lung maturation and surfactant production [8–13].

As a result of the critical roles that amniotic fluid plays in fetal development, analysis of this biofluid is used extensively in human clinical medicine for ante-partum assessment of fetal viability [1–4]. Amniocentesis is valuable in the prenatal detection of genetic defects, detecting fetal infections, monitoring lung maturity, and for gender determination in humans. However, there currently is not a detailed description of the lipidome for equine amniotic fluid. Therefore, to fully investigate the lipidomics profile of equine amniotic fluid we utilized a non-targeted high-resolution mass spectrometric approach. This experimental design minimizes bias and generates highly accurate lipid datasets. Using this research approach, we report, for the first time, the identification of cholesterol sulfate and amphiphilic (o-acyl) ω-hydroxy-fatty acids (OAHFA) in equine amniotic fluid. Amphiphilic OAHFAs have been

Abbreviations: ESI, electrospray ionization; DAG, diacylglycerol; FFA, free fatty acid; MS^2, tandem mass spectrometry; OAHFA, (o-acyl) ω-hydroxy-fatty acid; PlsC, choline plasmalogen; PlsE, ethanolamine plasmalogen; PtdC, phosphatidylcholine; PtdE, phosphatidylethanolamine; PtdG, phosphatidylglycerol; PtdI, phosphatidylinositol; TAG, triacylglycerol; VLCFA, very-long-chain fatty acid.

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identified previously in meibomian glands and meibum of humans, dogs, rabbits, and mice [14–19] and in equine sperm [20] but not in any other tissue or body fluid. Our data suggest that the surfactant properties of OAHFAs may play a unique role in the functions of amniotic fluid in supporting fetal development.

2. Materials and methods

2.1. Amniotic fluid collection and processing: pilot study

Amniotic fluid was collected from mares (N = 6) by ultrasound-guided transabdominal puncture as previously described [21]. Immediately after collection, recovered amniotic fluid was snap frozen in liquid nitrogen and stored at −80 °C until analysis. Identity of the recovered fluid as amniotic rather than allantoic fluid was confirmed based upon determination of Ca^{++}, Cl^{-}, and creatinine concentrations [22–23]. Mares were mixed light-breed, multiparous, with an average age of 9 years (range of 4–12 years).

2.2. Developmental study: amniotic fluid, amnion, maternal plasma, and fetal plasma collection and processing

Amniotic fluid, amnion, maternal plasma, and fetal plasma were collected from pony mares at 4 months (N = 4), 6 months (N = 4), and 9 months (N = 3) of pregnancy ±2 weeks. Samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis. The pony mares were an average age of 3.7 years (range of 3–5 years).

2.3. Lipidomics analyses of amniotic fluid

After thawing, 250 μL aliquots of amniotic fluid were mixed with 1 mL of methanol containing stable isotope internal standards [24–27]. Next 1 mL of water and 2 mL of methyl-*tert*-butyl ether were added and the tubes vigorously shaken at room temperature for 30 min. After centrifugation at 3000 × g for 10 min, 1 mL of the upper organic layer was dried by centrifugal vacuum evaporation prior to dissolution in 150 μL of isopropanol: methanol: chloroform (4:2:1) containing 15 mM ammonium acetate. Shotgun ESI lipidomics (5 μL/min) were performed utilizing high-resolution data acquisition (140,000 at 200 amu; 0.3–3 ppm mass error; *m/z* 200 to 1400) with an orbitrap mass spectrometer (Thermo Q Exactive).

In negative ion ESI (3.2 kV, capillary temp. of 320 °C, sheath gas of 10), the anions of ethanolamine plasmalogens (PlsEtn), phosphatidylglycerols (PG), (LPA), phosphatidylinositols (PtdIn), sterol sulfates, unsaturated fatty acids, dicarboxylic acids, and the $[M + HCOO]^-$ anions of ceramides were monitored. In positive ion ESI (4.3 kV, capillary temp. of 320 °C, sheath gas of 10), the cations of monoacylglycerols (MAG), choline plasmalogens (PlsCh), phosphatidylcholines (PtdCh), ceramides, and sphingomyelins (SM) and the ammonium adducts of diacylglycerols (DAG), and triacylglycerols (TAG) were monitored.

In the case of OHFAs, anions were quantitated and the identities of the fatty acid and hydroxy-fatty acids of individual OHFAs validated by MS^2 (Fig. 1) [20]. For MS/MS analyses, precursor ions were passed with unit mass resolution while the product ions were scanned utilizing high-resolution (140,000 at 200 amu; <2 ppm mass error) data acquisition.

Data are presented as R values (ratio of the endogenous lipid peak area to the peak area of an appropriate internal standard) per 250 μL of amniotic fluid (N = 6; mean ± SEM).

3. Results

3.1. Pilot study: choline glycerophospholipids (Table 1)

Equine amniotic fluid was found to be rich in diacylglycerophosphocholines (phosphatidylcholines, PtdC). Diacyl substitutions of PtdC with 30 or 32 carbons were mainly unsaturated fatty acids while PtdCs with diacyl substitutions of 34, 36, 38, 40, 42, or 56 carbons were mainly polyunsaturated fatty acids. Choline plasmalogens were much more limited in diversity in equine amniotic fluid.

3.2. Pilot study: ethanolamine, inositol, and glyceryl glycerophospholipids (Table 2)

Phosphatidylethanolamines, phosphatidylinositols, and phosphatidylglycerols were found to be present in equine amniotic fluid but with less diversity than choline glycerophospholipids. Ethanolamine plasmalogens, like choline plasmalogens, were less diverse than their diacyl counterparts. In the case of these lipids, polyunsaturated fatty acid substituents were minor.

3.3. Pilot sudy: sphingolipids (Table 3)

Equine amniotic fluid was found to possess limited sphingolipids, with SM d18:1/16:0 and Cer d18:1/16:0 being the major sphingomyelin and ceramide, respectively.

3.4. Neutral lipids (Table 4)

A number of diacylglycerols and a larger number of triacylglycerols were monitored in equine amniotic fluid. Diacylglycerol levels were significantly higher than triacylglycerol levels.

3.5. Pilot study: fatty acids (Table 5)

Amniotic fluid had a diverse array and high levels of free fatty acids. Also, in contrast to equine plasma, amniotic fluid had a large diversity of very-long-chain dicarboxylic acids but at significantly lower levels than free fatty acids.

3.6. Pilot study: OAHFAs and cholesterol sulfate (Table 6)

Strong molecular anions of OHFAs were monitored in negative ion ESI in equine amniotic fluid. The OAHFAs 48:2 and 50:2 had several isobaric forms with differing fatty acid and ω-hydroxy-fatty acid compositions, while only one major isobar was monitored for OHFA 51:2 and 52:3 (Table 6). However, these lipids also potentially have isobars that may have been masked by ion suppression. The predominant OHFAs are similar to those reported for meibum [14–19] and sperm [20] but with a more restricted diversity. The fatty acid constituents of these lipids were determined by MS^2 (Fig. 1) and found to include 16:1, 18:1, and 18:2, but not polyunsaturated fatty acids (Table 6). The ω-hydroxy-fatty acids were very-long-chain fatty acids (VLCFA) and included 30:0, 30:1, 32:1, 33:1, 34:1 (Table 6). Levels of the free forms of these VLCFA and their hydroxylated variants were not detectable in the organic extracts of 0.25 mL of amniotic fluid.

3.7. Developmental study (Fig. 2)

To monitor the timescale of OAHFA access to the amniotic fluid, samples were collected at 4, 6, and 9 months of gestation. Trace but measurable levels were monitored at 4 and 6 months but there was