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Analytical Methods

Glycosaminoglycanomic profiling of human milk in different stages of lactation by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Glycans in human milk serve several important biological functions that promote infant health. As kind of important glycans, glycosaminoglycans (GAGs) are a complex family of polyanionic carbohydrate, participating in a variety of critical physiological and pathological processes. In this study, the content and the detailed composition of human milk GAGs from Chinese mothers in different stages of lactation, based on a liquid chromatography-tandem mass spectrometry approach was investigated. The results showed that the GAG fraction in the human milk samples was very complex as it was composed of heparan sulfate, chondroitin sulfate, and hyaluronic acid. With lactation extending, the total amount of GAGs in human milk decreased. This study provided an important guide for the demands of GAGs during different stages of lactation. The results were also beneficial for studies on the composition and functional properties of infant formula.

1. Introduction

Human milk plays an integral role in the first period of life of newborns. Studies suggest that human milk not only provides nutrition, but also offer additional benefits such as stimulation of development and regulation of the newborn digestive system (German, Dillard & Ward, 2002), enhanced absorption of certain minerals, stimulation and function of the immune system (Rivero-Urgell & Santamaria-Orleans, 2001), and promotion of the development of the nervous system and brain (Morley & Lucas, 2000). Carbohydrates in milk perform a crucial function in the health of infants. Several types of human milk glycans, such as glycoproteins, glycolipids, and especially oligosaccharides, have been demonstrated to possess specific biological properties, and positively influence the health of breastfed newborn infants (Bode, 2012; Eidelman et al., 2012; Schanler, Lau, Hurst & Smith, 2005). However, only a few studies on human milk glycosaminoglycans (GAGs), a complex family of polyanionic carbohydrates, have been reported (Coppa et al., 2016; Coppa et al., 2011; Coppa, Gabrielli, Zampini, Bertino & Volpi, 2013; Coppa et al., 2012; Coscia et al., 2015; Newburg, Linhardt, Ampofo, & Yolken, 1995).

GAGs are linear natural polyanionic carbohydrates composed of repeating disaccharide units of uronic acid (glucuronic acid or iduronic acid) and amino sugar (glucosamine or galactosamine) (Gandhi & Mancera, 2008). GAGs are categorized into the following four classes on the basis of the type of monosaccharide composition, the kind of glycosidic linkage, the different of sulfated position and amount: hyaluronic acid (HA), galactosaminoglycans (chondroitin sulfate [CS] and dermatan sulfate [DS]), glucosaminoglycans (heparin and heparan sulfate [HS]), and keratan sulfate (Coppa et al., 2013; Tu, Ma, Bai & Du, 2017). The biological activities of GAGs are intimately related to their structural diversity. Quantitative disaccharide compositional analysis is one of the most important ways to characterize the structures of GAGs and has a direct relationship with their biological functions, including viral and bacterial infection and entry, angiogenesis, inflammation, cancer, and development (Linhardt, 2003).

The GAGs in the human milk were mainly composed of low-sulfated CS (~55%), HS (40%), and trace amounts of DS and HA. Previous studies demonstrated that GAGs in human milk serve as anti-infective agents by inhibiting the binding of the HIV envelope glycoprotein gp120 to the cellular CD4 receptor (Newburg et al., 1995) . Recently, a complete characterization and detailed evaluation of the GAGs of mature human milk and bovine milk were performed. Significant differences were found between human and bovine milk in terms of both quality and quantity (Coppa et al., 2011). Furthermore, Volpi et al.

Abbreviations: GAG, Glycosaminoglycan; HA, Hyaluronic Acid; CS, Chondroitin Sulfate; DS, Dermatan Sulfate; HS, Heparan Sulfate; HIV, Human Immunodeficiency Virus; gp120, Glycoprotein 120; CD4, Cluster of Differentiation 4; AMAC, 2-Aminoacridone; DMSO, Dimethyl Sulfoxide; LC-MS, Liquid Chromatograph-Mass Spectrometer; MRM, Mutiple Reaction Montitoring; pg, Picogram

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reported that the GAGs concentration in pooled milk from healthy mothers having delivered term or preterm newborns varied considerably during the first month of lactation, with the highest values being present in colostrum (Coppa et al., 2013). However, the detailed composition of GAGs in human milk and its dynamic variation in the different stages of lactation is still unclear.

This study evaluated the content and detailed composition of human milk GAGs from a Chinese mother in different stages of lactation (0–6 months) based on a liquid chromatography-tandem mass spectrometry approach. Further, the variation regularity was verified and assessed in different volunteer mothers. The detailed characterization of the glycan structure and composition during the development process were required to better understand the functions and mechanisms of the role of human milk in the process of infant development. These results can be also beneficial for studies on the composition and functional properties of infant formula.

2. Materials and methods

2.1. Materials

Unsaturated disaccharide standards—including unsaturated HA disaccharide (ΔUA-GlcNAc), unsaturated HS disaccharide standards (0S: ΔUA-GlcNAc; NS: ΔUA-GlcNS; 6S: ΔUA-GlcNAc6S; 2S: ΔUA2S-GlcNAc; NS2S: ΔUA2S-GlcNS; NS6S: ΔUA-GlcNS6S; TriS: ΔUA2S-GlcNS6S), and unsaturated CS disaccharide standards (0S: ΔUA-GalNAc; 2S: ΔUA2S-GalNAc; 6S: ΔUA-GalNAc6S; 4S: ΔUA-GalNAc4S; 2S4S/SB: ΔUA2S-GalNAc4S; 2S6S/SD: ΔUA-GalNAc6S; 4S6S/SE: ΔUA2S-GalNAc4S6S; TriS: ΔUA2S-GalNAc4S6S) were obtained from Iduron (Manchester, UK). *Escherichia coli* expression and purification of the recombinant heparin lyase I (EC 4.2.2.7), heparin lyase II (EC 4.2.2.X), heparin lyase III (EC 4.2.2.8), and chondroitin lyase ABC (EC 4.2.2.20) from *Flavobacterium heparinum* were performed as described previously (Shaya et al., 2006; Yoshida et al., 2002). Pronase was purchased from Roche (Basel, Switzerland). 2-aminoacridone (AMAC), $NaBH_3CN$, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of chromatographic grade from Sigma-Aldrich.

2.2. Milk samples

Human milk samples were obtained from nineteen mothers of newborns at 1–13 months postpartum and were included in the study after written consent. Human milk collection was approved by Ocean University of China. Breast milk at different stages of lactation, i.e. 12, 16, 22, 30, 43, 61, 88, 113, 140, and 183 days postpartum were provided by one of the mothers. The information on the demographic characteristics of the milk donors are shown in the supporting information (Table S1). All the samples were stored at −80 °C prior to testing.

2.3. Extraction and isolation of human milk GAG disaccharides

Extraction and isolation of GAGs from human milk samples were performed as described previously with some modifications, which in using ethanol to removal of lipids in milk samples rather than using chloroform, and some reagent amounts were varied according to the change in sample volume (Ninonuevo et al., 2008; De Leoz et al., 2013; Ninonuevo et al., 2006; Volpi, Galeotti, Yang & Linhardt, 2014; Wongtrakul-Kish et al., 2013). Briefly, each of the thawed milk samples was centrifuged (10 mL) at 4500*g* at 4 °C for 30 min, done in triplicate. The intermediate whey layer was collected, two volumes of ethanol were added to the whey layer, and the mixture was left at 4 °C overnight then centrifuged at 4500*g* at 4 °C for 10 min. The insoluble precipitate was collected, washed three times with ethanol, and dried to completion. The dried precipitate was individually subjected to proteolysis at 55 °C for 20 h with 10% pronase (20 mg/mL). After proteolysis, particulates were removed from the resulting solutions by passing each through a syringe filter containing a 0.22 μm membrane. Samples were then passed through Microcon centrifugal filter units (10 kDa) by centrifugation at 12,000*g* and washed with distilled water to remove peptides. The retentate was collected and lyophilized (Yang et al., 2011). Enzyme treatment and production of GAG disaccharides were performed as previously illustrated (Volpi et al., 2014). The lyophilized sample was dissolved in a digestion buffer (20 mM ammonium acetate, 1 mM calcium chloride, pH 7.0) and cocktail of chondroitin lyase ABC and heparin lyases (I, II, and III) were added. Then, the sample was incubated at 35 °C for 8 h to completely degrade the GAG samples. The reaction mixture was heated in a boiling water bath for 5 min. The denatured protein was removed by centrifugation at 12,000*g* for 10 min at room temperature. The supernatant was lyophilized to acquire the generated GAG disaccharide samples.

2.4. AMAC labeling

Human milk-derived GAG disaccharide samples together with unsaturated disaccharides standards were labeled by reductive amination with 2-aminoacridone (AMAC) (Yang et al., 2011). Disaccharide samples were reconstituted with 20 μL of 0.1 M AMAC solution in glacial acetic acid-DMSO (3:17, v/v) and 20 μL of freshly prepared solution of 1 M sodium cyanoborohydride in water was added in half-hour increments. Then, derivatization was performed by incubation at 45 °C for 4 h. Finally, the reacted mixture was diluted three times by the initial mobile phase and centrifuged to prepare the aliquots for LC-MS/MS analysis.

2.5. RP-HPLC-ESI-MS/MS analysis method

LC-MS/MS was performed with a TSQ Quantiva™ triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) running in multiple reaction monitoring (MRM) mode. HPLC separation was performed on a Luna C18 column (2 × 100 mm, 3 μm) at 45 °C with a gradient elution program at a flow rate of 140 μL/min. The mobile phases consisted of (A) 10 mM ammonium bicarbonate with the pH adjusted to 6 with acetic acid and (B) methanol. The gradient elution program was set at: 12% B (0–4 min), 12–25% B (4–25 min), 25% B (25–32 min).

Quantification analysis of AMAC-derivated disaccharides was performed based on the calibration curves constructed by increasing amounts of AMAC-derivated disaccharide standards (8, 40, 200, 1000, and 5000 pg per disaccharide). Linearity was assessed based on the amount of disaccharide and peak intensity in MRM mode. All analyses were performed in triplicate.

3. Results and discussion

3.1. Quantification analysis of GAG disaccharides

The LC-MS/MS GAGs analysis methods based on multiple reaction monitoring (MRM) are high sensitive and suitable for the analysis of the complicated biological samples without multi-step purification (Li et al., 2015). Hence, GAGs in human milk were first released and preliminarily purified based on proteolysis and ultrafiltration. Next, GAGs-derived disaccharides including HA, HS, and CS disaccharides, were generated by enzymatic digestion with a cocktail of heparin lyases I, II, and III and chondroitin lyase ABC. These disaccharides were AMAC-labeled by reductive amination and analyzed by LC-MS/MS using MRM. MRM could be used effectively for the analysis of a mixture of 16 AMAC-labeled disaccharide standards (Fig. 1A). The MRM of AMAC-labeled disaccharide standards and one of human milk samples is shown in Fig. 1. Seven types of HS-derived disaccharides, eight CS-derived disaccharides, and HA-derived disaccharides were detected.