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Altered breast milk components in preeclampsia; An in-vitro proton NMR spectroscopy study

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ABSTRACT

Objective: To investigate the metabolic profile of milk on day 3 and at the 6th month of lactation in mothers with preeclampsia (PE) and normotensive mothers.
Study design: Women with PE ($n = 29$) and control women ($n = 31$) were recruited for this study. Milk was collected on day 3 and at the 6th month of lactation. Proton NMR spectroscopy was used to identify 25 milk metabolites (alpha-lactose, beta-lactose, oligosaccharides, myo-inositol, alanine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine, valine, acetone, citrate, creatine, phosphocreatine, acetate, choline, lactate, lipid, phosphocholine and glycerophosphocholine). Principle component analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) were carried out to identify differences in milk metabolite composition between both the groups.
Results: The levels of milk metabolites varied between the control and PE groups. Alpha and beta-lactose, glycine, glycerophosphocholine ($p < 0.01$ for all); glutamate, glutamine and phosphocholine levels ($p < 0.05$ for all) were increased at the 6th month as compared to day 3 of lactation in the control group. However, in the PE group, only glycerophosphocholine level showed an increase ($p < 0.01$) at the 6th month. The levels of acetate, acetone ($p < 0.05$ for both) and creatine ($p < 0.01$) decreased at the 6th month as compared to day 3 of lactation in both groups. However, the levels of oligosaccharides were similar between groups and also similar at day 3 and at the 6th month of lactation.
Conclusion: Our data indicates differential levels of metabolites in the milk of women with PE. Future studies are required to investigate the associations between milk components and infant growth and development.

1. Introduction

Breast milk is the primary source of nutrition to the newborn. Milk provides various nutritional factors and other biologically active metabolites including sugars, amino acids, fatty acids and choline which are required for the development of the infant [1–5]. Milk metabolites either come from the diet or are synthesized in the mammary gland [6,7]. Reports indicate that the mammary gland development during pregnancy is influenced by angiogenesis [8]. Improper development of the mammary gland is known to affect lactogenesis [9]. Milk metabolite composition is also influenced by many factors such as maternal nutrition [10], stage of lactation [11] and pregnancy complications like preeclampsia (PE) [12,13]. Our earlier studies demonstrate altered angiogenesis in women with PE [14,15] which can influence mammary gland development and thereby lactogenesis [10,11].

Babies born to mothers with PE are associated with cognitive disorders in later life [16]. We have earlier reported lower growth in infants born to mothers with PE at six months of age [17]. It is likely that breast milk composition plays a critical role in influencing cognition [18] and overall growth [19]. It has been suggested that there are significant changes in the human milk metabolome at different stages of lactation indicating the nutrition received by the infant [20]. NMR-based metabolic profiling has been used to characterize different classes of human milk oligosaccharides (HMOs) to understand the nutritional properties of human milk [21]. Reports indicate higher oleic acid and linoleic acid in preterm formula milk as compared to preterm breast milk [22]. In contrast, higher lactose concentration in preterm breast

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milk as compared to preterm formula milk has also been reported [23]. Reports also indicate that there are differences in the levels of carnitine, caprylate, caprate, pantothenate, urea, lactose, oligosaccharides, citrate, phosphocholine, choline and formate in preterm milk as compared to full-term milk [24]. Higher choline and lower glycine p-cresol sulfate and hippurate have been reported in the urine of women with PE [25]. Women with PE are also reported to have higher plasma lysophosphatidylcholine 1 and lower lysophosphatidylcholine 2 and phosphatidylinositol as compared to normal pregnant women [26]. However, there are no reports on milk sugars, amino acids, fatty acid associated metabolites and energy metabolites in mothers with PE. The current study reports the levels of the above metabolites in milk on day 3 and at the 6th month of lactation using in vitro high-resolution proton (^{1}H) NMR spectroscopy in mothers with PE and compares them with normotensive (control) women.

2. Materials and methods

Subjects of the present study were recruited for a project funded by Indian Council for Medical Research (ICMR) (No. 5/7/396/09) from the Departments of Obstetrics & Gynecology and Pediatrics, Bharati Hospital, Pune during the year 2011–2012. This study was approved by the Institutional Ethical Committee (BVU/IRSHA/240/2014–2015). The current study includes women with PE ($n = 29$) and control women ($n = 31$). Written informed consent was taken from each subject. Subjects were excluded from the study if there was an evidence of other pregnancy complications like multiple gestations, chronic hypertension, type 1 or type 2 diabetes mellitus, seizure disorder, renal or liver disease. The control group consisted of pregnant women with no medical or obstetrical complications. PE was diagnosed by the criterion which has been discussed by us earlier [27]. Briefly, PE was defined by systolic and diastolic blood pressures ≥140 and 90 mm Hg respectively with proteinuria in a dipstick test. Birth measures like newborn weight and length were recorded at birth and 6 month of age.

2.1. Dietary assessments

A food frequency questionnaire (one month recall) was administered to estimate the frequency of consumption of various vegetarian food groups (cereals, vegetables, green leafy vegetables, fruits, milk products, pulses, oil type, and bakery products) and non-vegetarian foods (mutton, chicken, eggs and fish). Frequency of consumption of vegetarian and non-vegetarian food was analyzed using chi-square test.

2.2. Collection of milk samples

Human milk samples were collected on day 3 and at 6th month of lactation. Milk samples (2 mL) were collected into cryo vials and stored at −80 °C until further analysis. Milk samples were analyzed by NMR spectroscopy at the Department of NMR and MRI Facility, All India Institute of Medical Sciences, New Delhi.

2.3. NMR spectroscopy

For NMR spectroscopy, the milk samples were thawed and shaken thoroughly to homogenize the sample. Fifty microlitres of milk was mixed with 520 μL of D2O and formate (0.5 mM) was added to the sample that served as a concentration reference. Sodium trimethyl-silyl-[2, 2, 3, 3-H4] propionate (TSP) (0.5 mM) was added to the sample, that served as a chemical shift reference for the proton NMR studies. It has been reported that TSP interacts with the proteins (such as albumin) present in biological samples [28]. Alpha-lactalbumin (albumin present in milk) is the major protein in breast milk, which may bind with TSP and thus may influence the accurate quantitative determination of metabolites [29]. Therefore, in this study, formate was used as concentration reference while TSP was used only as the chemical shift reference. The ^{1}H NMR experiments of milk samples were performed on 700 MHz spectrometer (Agilent Technologies, Santa Clara, U.S.A.). One-dimensional (1D) ^{1}H spectrum with water suppression were acquired with a single 90° pulse. The typical parameters for 1D experiment were: spectral width of 9124.1 Hz, data points 32K, number of scans 16. Since, the longitudinal relaxation time (T1) value of formate is 12.7 s at 700 MHz, a relaxation delay of 70 s was used to attain complete relaxation for appropriate quantification of metabolites. The data was processed on a Dell 390N, PC, Red Hat Enterprise Linux workstation using the Varian software, Vnmrj 3.2A (Agilent Technologies, Santa Clara, U.S.A.). The free induction decays were multiplied by an exponentially decaying function prior to Fourier transformation. The value of LB is 0.3 Hz. Spectra were manually phased using zero and first order corrections. Water resonance was suppressed using the pre-saturation pulse at the water frequency.

Two-dimensional (2D) double quantum filtered correlation spectroscopy (DQF COSY) and total correlation spectroscopy (TOCSY) experiments were also carried out at 700 MHz for few samples for unambiguous assignment of various resonances. This helps in identifying the complete networks of spin couplings in a molecule. TOCSY experiment was carried out using the following parameters: 2K data points in F2 dimensions, spectral width of 9124.1 Hz, relaxation delay of 2 s and mixing time of 80 ms, 200 time domain points (number of experiments) were collected in t1 with 16 acquisitions. The parameters for 2D DQF COSY experiments were 2K data points in F2 dimensions, spectral width of 9124.1 Hz, relaxation delay of 2 s and 200 time domain points (number of experiments) were collected in t1 with 16 acquisitions.

2.4. Quantification of the metabolites

The concentration of the metabolites was determined by comparing the integrated intensity of isolated resonance of the compounds of interest with that of the formate signal. In this study, we could not determine concentration using Chenomx software as it uses, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) or 3-(trimethylsilyl) propionic acid (TSP) for the quantification of metabolites. A total of 14 metabolites (alpha-lactose, beta-lactose, alanine, glutamate, glutamine, glycine, acetate, choline, phosphocholine, glycerophosphocholine, acetone, citrate, creatine, and phosphocreatine) were quantified from NMR spectra of human milk samples. These metabolites were classified as sugars (alpha-lactose and beta-lactose), amino acids (alanine, glutamine, glutamate, and glycine), fatty acids and their metabolites (acetate, choline, phosphocholine, glycerophosphocholine) and energy metabolites (acetone, citrate, creatine and phosphocreatine).

2.5. Statistical analysis

The data were analyzed using SPSS/PC + package for MS Windows (Version 20, Chicago, IL). Levels of various milk metabolites were expressed as mean ± SD. The 'Shapiro–Wilk' test was used to test the variables normality distribution. Skewed variables were transformed to satisfy the underlying assumption of normality using natural logarithm transformation (Loge). Mean values of the estimates of milk metabolites were compared by Student's "*t*" test. The degree of significance is denoted by the value of *p*, and is considered statistically different when $p \leq 0.05$.

2.5.1. Multivariate data analysis

Principle component analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) was also carried out on the binned data of the ^{1}H NMR spectrum to discriminate between samples of diseased and healthy subjects using Unscrambler 10.2 (CAMO, Oslo, Norway). The ^{1}H NMR spectra were binned by subdividing into 0.02 ppm regions using the Chenomx NMR Suite 7.5 software (Chenomx Inc. Edmonton, Canada) to reduce the spectrum into 200 variables in the region of