Intestinal Barrier Maturation in Very Low Birthweight Infants: Relationship to Feeding and Antibiotic Exposure

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Objective To test the hypothesis that feeding and antibiotic exposures affect intestinal barrier maturation in preterm infants, we serially measured intestinal permeability (IP) biomarkers in infants <33 weeks gestation (gestational age [GA]) during the first 2 weeks of life.

Study design Eligible infants <33 weeks GA were enrolled within 4 days of birth in a prospective study of IP biomarkers (NCT01756040). Study participants received the nonmetabolized sugars lactulose/rhamnose enterally on study days 1, 8, and 15 and lactulose/rhamnose were measured in urine by high-performance liquid chromatography. Serum zonulin and fecal alpha-1-anti-trypsin, 2 other IP markers, were measured by semiquantitative Western blot and ELISA, respectively.

Results In a cohort of 43 subjects, the lactulose/rhamnose ratio was increased on day 1 and decreased over 2 weeks, but remained higher in infants born at ≤28 weeks of gestation compared with IP in infants born at >28 weeks of gestation. Exclusive breastmilk feeding was associated with more rapid maturation in intestinal barrier function. A cluster analysis of 35 subjects who had urine samples from all time points revealed 3 IP patterns (cluster 1, normal maturation: n = 20 [57%]); cluster 2, decreased IP during the first week and subsequent substantial increase: n = 5 [14%]); and cluster 3, delayed maturation: n = 10 [29%]). There were trends toward more prolonged antibiotic exposure ($P = .092$) and delayed initiation of feeding ≥4 days ($P = .064$) in infants with abnormal IP patterns.

Conclusions Intestinal barrier maturation in preterm infants is GA and postnatal age dependent, and is influenced by feeding with a maturational effect of breastmilk feeding and possibly by antibiotic exposures. *(J Pediatr 2017;183:31-6).*

Trial registration ClinicalTrials.gov: NCT01756040.

Necrotizing enterocolitis (NEC), a life-threatening, gastrointestinal emergency affects approximately 7%-10% of very low birthweight preterm neonates[1,2] with mortality as high as 30%-50%.[3] Although breast milk has been shown to be protective against NEC,[4,5] postnatal antibiotic exposure may increase the risk for NEC.[6,7] Intestinal barrier immaturity is the proximate cause of susceptibility to NEC in preterm neonates,[8,9] but few preterm infants born at <30 weeks of gestation have been included in prior studies of intestinal permeability (IP)[10-15] and the impact of current feeding practices and antibiotic exposures on intestinal barrier maturation in the extremely preterm population is unknown.

The percent urinary excretion of orally administered isotonic solutions of the nonmetabolized sugars lactulose and rhamnose as markers of the intestinal paracellular and transcellular pathways, respectively, is the gold standard to assess IP. These tests have been used extensively for >30 years to assess IP in adults[16-18] and in preterm[10-15] and term infants,[13,19,20] as well as in older children. The sugar probes have been used safely to assess IP in newborns with birth asphyxia,[10] NEC,[11] and congenital heart disease.[20,21] Other markers of impaired intestinal barrier function include zonulin in adults[22] and alpha-1-anti-trypsin (A1AT) in children.[23] Zonulin is a 47-kDA eukaryotic cellular protein that regulates the intestinal epithelial paracellular pathway by reversibly opening mature tight junctions[24] and is up-regulated in several autoimmune diseases, including celiac disease and type 1 diabetes.[22] Whether zonulin is involved in tight junction maturation in preterm infants is unknown. Determination of fecal A1AT is used routinely in clinical practice as an indicator of substantial increased IP and protein-losing enteropathy.[23]

To test the hypothesis that feeding and antibiotic exposures modulate intestinal barrier function in preterm infants, we conducted a prospective study to measure

A1AT	Alpha-1-anti-trypsin	La/Rh	Lactulose/rhamnose
GA	Gestational age	NEC	Necrotizing enterocolitis
IP	Intestinal permeability		

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IP biomarkers (urinary lactulose/rhamnose [La/Rh] ratio, serum zonulin, and fecal A1AT) serially in infants born at <33 weeks of gestation (gestational age [GA]) during the first 2 weeks of life.

Methods

All admissions to the University of Maryland Medical Center and Mercy Medical Center NICUs who were $24^{0/7}$-$32^{6/7}$ weeks gestation <4 days of age were screened for study eligibility and parental consent of eligible subjects was obtained (ClinicalTrials.gov: NCT01756040). The institutional review boards of both institutions approved the study. Exclusion criteria included nonviability or planned withdrawal of life support; triplet or higher order multiples; severe asphyxia (Apgar score < 3 at 5 minutes and cord pH < 7.0); lethal chromosomal abnormalities; cyanotic congenital heart disease; intestinal atresia or perforation; abdominal wall defects; significant gastrointestinal dysfunction (eg, heme-positive stools, abdominal distension (girth > 2 cm baseline) or bilious emesis/aspirates, and infants with galactosemia or other forms of galactose intolerance. Before study procedures, a complete physical examination including vital signs, weight, height, and head circumference was performed. Demographic, clinical, and adverse events data were collected from the medical record.

Both participating clinical centers used the same standardized feeding protocol. Feeds were initiated between the first and fourth days of life depending on clinical stability. After initial feeds of 10 mL/kg expressed breast milk or 20 kcal/oz preterm formula daily for 3-5 days, feedings were advanced by 20 mL/kg/d until 100 mL/kg/d was reached. Subsequently, caloric density was advanced to 24 kcal/oz before increasing feeding volume by 20 mL/kg/d to 150 mL/kg/d. Feedings were held or discontinued for signs of feeding intolerance, such as abdominal distension, gastric residuals, or hematochezia, or for clinical deterioration.

Lactulose/Rhamnose Sugar Absorption Test and Sample Collection

On each of the 3 study days (days 1, 8 ± 2, and 15 ± 2), participants received 1 mL/kg La/Rh solution (8.6 g of lactulose [Cumberland Pharmaceuticals, Nashville, TN] + 140 mg of rhamnose [Saccharides, Inc, Calgary, Alberta, Canada]/100 mL) by nipple or by gavage via a clinically indicated orogastric tube.[12] A minimum of 2 mL of urine was collected over a 4-hour period after the La/Rh dose was administered, with cotton balls placed in the infants' diaper. The total urine volume recorded included the volume extracted from the cotton balls in addition to the estimated volume of urine that leaked into the diaper determined by the diaper weight. The test was repeated the next day if <2 mL urine was collected. If there were signs of feeding intolerance (increased abdominal girth > 2 cm, heme-positive stools, or gastric residuals), the sugar solution administration was either delayed until resolution of symptoms within the study four day window for each timepoint or not done. Serum (total 0.5 mL) was collected by heel stick 90-120 minutes after La/Rh dosing to measure La/Rh[25] and serum zonulin. A stool sample (~1 g) was collected within 8 hours of the sugar probe dosing for A1AT analysis. Urine, serum, and stool samples were stored at −80°C until analysis. The amount of lactulose and rhamnose in each sample was measured using high-performance liquid chromatography and adjusted for urine volume.[26] The fractional urinary excretion of lactulose and rhamnose was calculated as the ratio of the total urinary excretion of each sugar probe to the total oral dose of the probe. For each subject, the lactulose/rhamnose ratio (La/Rh ratio) was calculated as the fractional excretion of lactulose divided by that of rhamnose.[27] A La/Rh ratio of >0.05 was considered indicative of increased IP.[10]

Serum Zonulin Western Blot

Serum samples (70 μg per well) were run under nondenaturing conditions on 4%-20% Tris-Glycine gels (Invitrogen, Waltham, Massachusetts). Protein was transferred onto a PVDF membrane (Millipore, Billerica, Massachusetts) and probed with 1.5 μg/mL mouse monoclonal zonulin antibody (Bio-Rad, Hercules, California). Bands were detected with Alexa Flor 680 conjugated goat anti-mouse IgG antibodies (ThermoFisher, Waltham, Massachusetts). Bands were visualized and densitometry was measured using Image Studio software (LI-COR Biosciences, Lincoln, Nebraska). All samples were normalized to a healthy term control reference sample run separately on each gel.

Stool A1AT ELISA

Stool samples diluted 1:250 according to the manufacturer's protocol were analyzed by double sandwich LISA (Eagle Biosciences, Nashua, New Hampshire) and results expressed as micrograms per gram of stool.

Statistical Analysis

The La/Rh ratio, serum zonulin, and stool A1AT data are represented as the mean and SD at each of the 3 time points. Categorical data were compared using the χ^2 test and continuous data were compared with the Student t test. To quantify the association between urine La/Rh ratio, serum zonulin, and stool A1AT, Pearson correlation coefficients between the gold standard urinary La/Rh ratio and each of the other IP measures were calculated. IP patterns were differentiated using cluster analysis based on Ward minimum variance method, as implemented in SAS 9.3 (SAS Institute, Cary, North Carolina).

Results

Forty-four subjects were enrolled over an 18-month period from April 15, 2013, to October 15, 2014, and 43 subjects received ≥1 dose of sugar solution (**Figure 1**; available at www.jpeds.com) (ClinicalTrials.gov: NCT01756040). Demographic characteristics of the participants are represented in the **Table**. Because we were interested in the maturation of