Characteristics of the Meconium Microbiota in Neonates with Fetal Growth Restriction

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Abstract

Background

Fetal growth restriction (FGR) is a major cause of fetal and neonatal morbidity and mortality. Since meconium microbiome is a critical component for the health of newborns, the aim of this study was to characterize the meconium microbiota in neonates with FGR compared with healthy controls.

Methods

To avoid bacteria contamination, all included neonates were delivered by elective C-section, with their samples collected in the operation room. We collected meconium samples from 14 neonates affected by FGR and 20 matched healthy newborns. The microbiome profiles were examined by using high-throughput 16S rRNA sequencing and analyzed with customized bioinformatics pipelines.

Results

The β -diversity analysis demonstrated that FGR neonates clustered together that was distinct from control subjects. A number of taxa were found to be significantly depleted (e.g., *Chryseobacterium*) or enriched (e.g., *Asticcacaulis*) in the FGR group. In addition, the coordination network between various intestinal bacteria was found to be altered in FGR neonates.

Conclusions

FGR neonates exhibited alterations in the composition and symbiosis of microbiome, which will contribute to a better understanding of the relationship between the gut microbiota and FGR.

Introduction

Fetal growth restriction (FGR) is confused with small for gestational age (SGA) in clinical practice. ACOG guidelines issued in 2019 directly define FGR as an estimated fetal body weight (EFW) that is less than the 10th percentile for gestational [1], whereas RCOG and SOGC guidelines regard it as the diagnosis of SGA [2]. According to RCOG and SOGC guidelines, FGR is a pathologic condition in which the fetus fails to reach its biological growth potential. Some, but not all, growth restricted fetuses/infants are SGA while 50–70% of SGA fetuses are physiologically small, with fetal growth appropriate for maternal size and ethnicity [3]. The fetus with the lower EFW percentile is more likely to be diagnosed as FGR after birth [4]. One third of all stillbirths occur at term and infants with a birth weight lower than the third percentile at term have an eightfold increased risk of antepartum stillbirth [5]. The World Health Organization (WHO) defines FGR as an EFW below the third percentile, which was also used as the inclusion criteria of our study group in order to avoid including some constitutionally normal newborns.

FGR is a major cause of fetal and neonatal mortality and morbidity, affecting about 8% of all pregnancies [6]. More importantly, neonates affected by FGR are more susceptible to metabolic disturbance (e.g., glucose and fatty acid metabolism disturbance), thereby increase the risks of developing chronic metabolic diseases in adulthood. FGR is determined by a complex interaction of various factors, which can be broadly categorized into maternal, fetal and placental. Although the primary pathophysiologic mechanisms underlying these conditions are different, they often (but not always) have the same final common pathway: sub-optimal uterine–placental perfusion and fetal nutrition [1]. However, there is no high-quality evidence to suggest that additional nutrient intake in the absence of true maternal malnutrition increases fetal weight or improves the outcome in the cases of suspected FGR [7]. To date, there is still no effective treatment for FGR in clinical practice.

Metagenomic analysis of the infant microbiome suggested that intestinal microbiome may play important roles in the nutrition absorption by participating in energy harvest from the diet and to modulating host energy storage and metabolism [8]. Li et al. reported that probiotic supplement could change the gut microbial community of very low birth weight (VLBW) infants, suggesting potentially correlation between gut microbiome and infant growth [9]. However, the association between meconium microbiota composition and FGR newborn remains poorly understood.

Here we performed a case-control study using meconium samples collected from 14 FGR and 20 healthy newborns. High-throughput 16S rRNA gene sequencing and bioinformatics analysis were applied to identify altered meconium microbiota associate with FGR.

Materials And Methods

Study subjects

During June 2019 to April 2020, pregnant women who planed to receive elective caesarean section at Affiliated Shenzhen Maternity & Child Healthcare Hospital, Southern Medical University were invited to participate in this study. The inclusion criteria were: 1) pregnant women in study group whose an EFW was less than the 3th percentile; 2) pregnant women in control group whose an EFW was between the 10th to 90th percentile; 3) singleton pregnant women; 4) pregnant women without any complications, except for advanced maternal age, abnormal presentation, scar uterine pregnancy, pregnancy associated with hysteromyoma; 5) pregnant women who received ultrasound between 8–14 weeks; 6) normal result of the non-invasive prenatal test or invasive prenatal diagnosis; 7) The fetuses were excluded fetal malformation by morphology ultrasound in tertiary hospital. The exclusion criteria were: 1) The neonatal birth weight did not meet inclusion criteria; 2) threatened labor or in labor; 3) preoperative fasting < 8 hours; 4) pregnant women using any type of antibiotic or probiotic within a period of 2 months before delivery; 5) newborns with malformation or chromosomal abnormalities within 1 month after birth; 6) cord blood sample hemolysis after centrifugation. In total, 34 newborns involving the final analysis were divided into FGR group (n = 14) and the control group (n = 20). The sampling protocol and research proposal were approved by the Ethical Committees of Affiliated Shenzhen Maternity & Child Healthcare

Hospital, Southern Medical University. All mothers were made aware of the details of the study before obtaining written informed consent.

Meconium sample collection and DNA extraction

All meconium samples and umbilical blood samples were collected in our operation room by the first author. To avoid bacteria contamination, all sampling operations followed the principle of sterility. Rectal swabs (CY-98000, HCY Technology, Shenzhen, China) were obtained by inserting into the rectum (to a depth of 4 cm) and rotating by 360°, while scrapping the rectal wall for no more than 5 seconds to avoid discomfort. Once collected, the swab tip was snapped off into a 1.5 mL sterilized centrifuge tube containing preservation solution (CY-F002-10, HCY Technology, Shenzhen, China). These samples were immediately stored at -80°C until DNA extraction. DNA from stool samples was extracted using QIAamp Fast DNA Stool Mini Kit according to manufacturer's instructions (Qiagen, Germany). All experiments were carried out on a sterile bench. DNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE).

Cord blood sample collection and measurement

The cord blood sample (3 mL) was drawn immediately after delivery and centrifuged at 3000 rpm for 10 min to separate serum for the measurements. Plasma glucose was measured by glucose oxidase method using Beckman Coulter UniCel DXC 800 Synchron™ Clinical Systems. Plasma insulin was measured by chemiluminescent enzyme immunoassay using Beckman Coulter Dxl-800 analyzer.

PCR amplification and pyrosequencing

The V3-V4 region of 16S rRNA gene was amplified using the forward primer (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer (5'-GGACTACHVGGGTWTCTAAT - 3'). The normalization of PCR product was performed using the SequalPrep Normalization Plate Kit (Life Technologies). Pooling and sequencing were conducted on the Illumina MiSeq platform using a dual-indexing strategy [10].

Data analysis

The relative abundance for each bacterial level from phylum to genus was measured using QIIME pipeline. The Chao, Ace, Shannon and Simpson indexes were calculated to assess α -diversity within group. The β -diversity was assessed by unweighted Unifrac distance matrix and visualized by NMDS (Non-metric multidimensional scaling) plot. LEfSe (linear discriminant analysis effect size) tool was used to discover the features contributing to the most variation between control and FGR groups.

SparCC analysis was performed with default parameters and 1000 bootstraps to test for correlations between the relative abundances of genera [11]. Pseudo P-values were calculated as the proportion of simulated bootstrapped data sets with a correlation at least as extreme as the one computed for the

original data set. The cutoff for statistical significance was set at pseudo $P < 0.01$. The co-occurrence network of species was visualized in Cytoscape software [12].

Statistical analyses were performed using R software (version 3.6.1). Continuous variables were reported as means $\pm$ standard deviations. Student's t-tests were used to study differences in continuous variables. P -value < 0.05 was considered statistically significant.

Results

Basic characteristics of study subjects

A total of 14 FGR and 20 normal newborns were included in this study. As expected, the birth weight and gestational age at delivery were significantly lower in FGR group than that in control group. On the other hand, maternal age, maternal pre-pregnancy body mass index (BMI), maternal weight gain, plasma glucose and insulin level did not differ significantly between the two groups (Table 1).

Table 1
Clinical variables of FGR and control neonates

	FGR (n = 14)	Control (n = 20)	P-value
Maternal age (year)	32.00 $\pm$ 5.15	32.30 $\pm$ 4.19	0.853
Maternal pre-pregnancy BMI (kg/m2)	19.92 $\pm$ 1.88	21.25 $\pm$ 2.02	0.061
Maternal weight gain (kg)	12.31 $\pm$ 3.65	13.89 $\pm$ 4.02	0.251
Gestational age at delivery (wk)	37.55 $\pm$ 0.93	39.27 $\pm$ 0.68	< 0.001
Birth weight (g)	2231.43 $\pm$ 140.87	3314.50 $\pm$ 295.68	< 0.001
Glucose level (mmol/L)	3.25 $\pm$ 0.40	3.49 $\pm$ 0.36	0.084
Insulin level (pmol/L)	34.35 $\pm$ 30.40	51.82 $\pm$ 40.33	0.199

Meconium microbiome composition of FGR

The α -diversity of meconium microbiome was compared between the newborns in the FGR and healthy groups. As compared to healthy controls, a trend towards lower diversity measured by Chao and Ace indexes was found in FGR neonates (Fig. 1A and 1B, Chao $P = 0.047$, Ace $P = 0.0098$). However, microbial diversity represented by Shannon and Simpson indexes indicated no significant difference between the two groups (Fig. 1C and 1D).

In addition, the unweighted UniFrac distance between individual samples was calculated to estimate the β -diversity in microbial communities. The heatmap showed an evident clustering for FGR subjects (Fig. 2A). And NMDS plot further revealed the differences between two groups (Fig. 2B, PERMANOVA P -value = 0.033), as the FGR samples tended to assemble in the coordinate diagram.

Differentially abundant taxa between FGR and normal groups

The LEfSe software was used to further analyze bacterial communities (see Materials and Methods), so as to identify differentially abundant taxa between the FGR and normal infants. We observed significant differences at family and genus levels. In particular, a list of genera including *Vibrionimonas*, *Asticcacaulis* and *Labrenzia* were relatively more abundant in the FGR group, whereas genus *Chryseobacterium* was more enriched in the control group (Fig. 3).

SparCC analysis (see Materials and Methods) was performed to gain deeper insight into the correlative bacterial populations at genus level (Fig. 4). Notably, a series of genera were found to be connected to form a cluster in the gut microbiome of control subjects, while only a few genera were loosely clustered in the FGR samples. Such apparent distinction in terms of network structure suggested potentially altered microbiota function in FGR condition (see Discussion below).

Discussion

In this study, we demonstrated that meconium of FGR infants possessed distinct gut microbiota as compared with healthy infants. And the altered FGR-related microbial community was characterized by the reduced abundance of *Chryseobacterium* genus and impaired connections between genera. These findings may contribute to the ongoing exploration on the potential role of microbiota regulation in FGR, thus providing clues for development of potential preventive and treatment measures.

As measured by various indexes, the α -diversity of neonates with FGR did not show consistent difference to that of healthy ones. A number of studies have suggested that the intestinal ecosystem of neonates is characterized by unstable microbial composition, which was profoundly influenced by multiple factors such as delivery mode and type of feeding [13, 14]. And microbiome investigation on placenta samples also indicated no substantial difference in α -diversity between FGR and normal subjects [15]. Therefore, α -diversity may not represent the major characteristics of gut microbiota in FGR neonates.

In addition, FGR and control groups could be differentiated in the β -diversity analysis, as FGR samples were clustered together in heatmap and NMDS plot. In particular, *Chryseobacterium* was found to be decreased in FGR neonates. As a gram negative and rod-shaped bacteria, *Chryseobacterium* belongs to *Flavobacteriaceae* family [16] and widely exists in natural environments, plants, and animals [17]. The *Chryseobacterium* genus is normally harmless and plays an important role in metabolism of amino acids and carbohydrate [18]. The functional annotations suggested that the lack of *Chryseobacterium* in infant gut microbiome may affect nutrient uptake during perinatal period.

The symbiosis in normal microbiome involves the coordination between various intestinal bacteria. Previous studies have indicated that such coordination might be compromised in pathological condition [19]. Likewise, our results showed that the functional cluster of bacteria in normal neonates seemed to be undermined in FGR subjects. However, the detailed mechanisms underlying the interaction between different bacteria in the human gastrointestinal tract remain to be determined.

However, the study is also subjected to several limitations. Firstly, the sample size was not very large and all the participants were recruited from the same hospital, hence we cannot completely rule out the potential regional differences in placental microbiota. Secondly, we were not able to record detailed information on lifestyle of the mothers during the course of pregnancy, so the effect of dietary intakes and microbial transmission from mother to child were not thoroughly analyzed. We plan to address the above issues in our subsequent research with expanded cohort.

In summary, we found that FGR infants had distinct gut microbiota as compared with healthy controls. Our results provided insights into potential connections between the neonatal intestinal microbiome and the outcome of FGR, which is important for improving infant health. Thus, high-quality pre-clinical experiments and large-scale clinical studies are urgently needed to further clarify the underlying mechanisms.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethical Committees of Affiliated Shenzhen Maternity & Child Healthcare Hospital, Southern Medical University..

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XT, QZ and JY were major contributors of study design. XT performed the sample collection. CD and BL performed laboratory testing. KL, JG and HY performed the data analysis. XT and KW contributed in writing the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Comparison of α -diversity between the meconium microbiota of FGR cases and controls. Four indexes were calculated to represent the α -diversity (A, Chao index; B, Ace index; C, Shannon's diversity index; D, Simpson's diversity index).

Figure 2

Clustering of gut microbiota profiles based on the unweighted UniFrac distance between individual samples. (A) Heatmap and (B) NMDS plot illustrated distinction between FGR and control groups.

Figure 3

Differentially abundant microbial taxa. (A) Cladogram representation of gut microbiota taxa from the phylum level down to the genus level. (B) LDA scores calculated for differentially abundant taxa in two groups. Taxa at family, genus and species level were denoted with label [5], [6] and [7], respectively.

Figure 4

Correlation network between bacteria in FGR (A) and control (B) samples, with nodes representing bacterial genera (colored according to phylum, size corresponds to mean relative abundance) and edges representing significantly positive (pink) or negative (blue) correlations.