Fetal echogenic bowel may be related to intestinal microbiota: a prospective cohort study

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Abstract

Objective: The purpose of the current study was to determine the difference in intestinal microbiota after delivery between healthy fetuses and fetuses with hyperechogenic bowel during the second trimester and the relationship between fetal echogenic bowel and microbiota. Design: Prospective cohort study. Setting: The Second Affiliated Hospital Of Fujian Medical University,Quan zhou, China. Population: 34 single pregnant women and their fetuses. Methods: Fourteen healthy fetuses (control group), 13 fetuses with echogenic bowel (EB group), and 7 fetuses with echogenic bowel and other abnormalities (C-EB group) were selected. The first meconium after delivery was collected for 16s rRNA sequencing. Results: A total of 69,222 operation classification units were obtained by clustering all high-quality sequences with 97% similarity. There was no significant difference in the Shannon, Simpson, and Chao 1 indices among the three groups. At the phylum level, the intestinal microflora of the three groups were similar.At the genus level, the abundance of Escherichia coli/Shigella in the EB、C-EBgroup was significantly lower than the control group, while the abundance of Staphylococcus, Methylobactrium, and Curvibacter in the EB group was significantly higher than the other groups. There was a difference in abundance of Gammaproteobacteria, Fusobacteria, Enterobacteriaceae, and E. coli in the EB and C-EB groups. Conclusion: The intestinal flora may be related to fetal echogenic bowel via mucus secretion and intestinal gas production.

Introduction

Fetal echogenic bowel (FEB) refers to the ultrasound images of the fetal intestine, which are similar to or stronger than the echo of adjacent bone. [1] FEB is one of the soft indices of fetal chromosomal abnormalities. Between 0.2% and 1.8% of fetuses have this sign in the second trimester of pregnancy based on an ultrasound examination.[2-3] Most fetuses with FEB have a good outcome, but some fetuses have an underlying pathologic condition, including chromosomal abnormalities, cystic fibrosis (CF), intrauterine growth restriction (IUGR), and intrauterine cytomegalovirus.[4] However, The mechanism of FEB is unclear. Some studies suggest that meconium may be caused by fetal intestinal mucus, intestinal gas, and ingestion of amniotic fluid.[5-8]

The traditional belief has always been that the fetus is sterile*in utero* .[9] With the development of modern sequencing technology, more and more evidence has shown that the fetus is colonized *in utero* . Collado et al.[10]reported a low abundance microbiome in the amniotic fluid and placenta. Several research teams have shown that bacteria not only exist in the fetal environment of healthy term pregnancies, but also constitute the placental microbiota that participate in normal fetal development and initiate colonization of the fetal microbiota.[11-14] de Goffau et al.[15] concluded that the microbiota is transferred between the maternal and fetal interface. Martinezka et al.[16] found that there are rich and diverse bacterial DNA in mouse embryo

guts. The bacterial DNA is transferred from the maternal microbiota to the fetal gut and may be the key stimulating factor to promote normal development of fetal mucosal immunity. A recent radioimmunoassay study showed the presence of culturable bacteria in the fetal gut in the second trimester.[17] Such evidence indicates that colonization of human intestinal flora begins in the fetal period.

In recent years, a number of studies have shown that several diseases during the fetal period are related to intestinal flora. Guzzardi et al.[18] found that growth of the fetal heart is associated with colonization of the intestinal flora *in utero* . Zhang et al.[19] found that the diversity of the intestinal flora in newborn piglets with IUGR was decreased. Therefore, we propose a hypothesis that hyperechogenic bowel may be related to intestinal flora via mucus and gas. The purpose of this study was to determine the intestinal flora of neonates using high-throughput sequencing technology, as well as the relationship between hyperechogenic bowel and intestinal flora.

Materials and Methods

Fourteen fetuses without apparent abnormalities were selected as the control group. Thirteen fetuses diagnosed with isolated hyperechoic bowel, but without serious malformations and chromosome abnormalities were selected as the EB group from December 2018 to September 2019. At the same time, seven fetuses diagnosed with hyperechoic bowel and other abnormalities were selected as the C-EB group. Group C-EB Included one case with an isolated lung, one case with a persistent left superior vena cava and coronary sinus dilation, one case with a right subclavian artery vagal with renal pelvis separate, one case with a neck hemangioma, one case of a premature infant, one case with left hydronephrosis, and one case with right subventricular cyst.

The inclusion criteria for the control group were as follows: 1) singleton full-term newborn delivered via caesarean section; 2) fetal prenatal ultrasound showed no strong echoes of the intestinal tract, no other abnormalities, and the gestational age by ultrasound was consistent with the actual gestational age; 3) all prenatal data available; and 4) normal maternal menstrual pattern, good maternal physical health, no antibiotics used in the first 3 months of pregnancy, no maternal smoking, no maternal history of diabetes or heart disease, no abnormalities on maternal biochemical and Torch testing.

The inclusion criteria for the EB group were as follows: 1) according to the Slotnick and Abuhamad ultrasonographic grading system, all cases in this group were $>$ grade 2 without any other abnormalities; and 2) the inclusion criteria were otherwise the same as the control group.

The inclusion criteria for the C-EB group were as follows:according to the Slotnick and Abuhamad ultrasonographic grading system, the patients in this group were $>$ grade 2 with other abnormalities; and 2) the inclusion criteria were otherwise the same as the control group.

The exclusion criteria were as follows: 1) the mother had ingested a probiotic preparation within 1 month before delivery; 2) the newborn was fed milk before the fecal collection; and 3) the mother participated in another clinical trial at the same time.

The clinical data from the parturient were collected as follows: parturient age; gestational age; and mode of delivery. The clinical data from the infants were collected as follows: height; weight; gender; and Apgar scores at 1, 5, and 10 min.

AGE Voluson E8 and E10 Doppler ultrasound machines(Voluson E8 and E10,GE,Boston, Massachusetts,USA) were used to evaluate fetal growth, the amniotic fluid volume, placenta, and umbilical cord. Then, we evaluated the echo intensity of fetal intestines and iliac crest. Hyperechogenic bowel was diagnosed by two senior physicians at the same time. The echo intensity of the fetal intestines was the same as or stronger than that iliac bone on the same sonogram (Figure 1).

Sample Collection and DNA Extraction

Meconium was collected from newborns who had not been weaned within 2 hours after birth, and was labeled control, EB, and C-EB groups. While wearing sterile gloves, the central part of fresh feces was probed and

sampled with a sterile cotton swab the size of a soybean (3-5 g), placed into a 2-mL sterile storage tube(Stool collection tube,Zhejiang Gongdong Medical Equipment Co.,Ltd,Zhe jiang ,China), and immediately place into an -80°C freezer for storage.

DNA was extracted from fecal samples according to the instructions of a Fecal Bacteria Genomic DNA Extraction Kit (centrifugal column type,Beijing Tiangen Inc, Beijing, China), then stored in a freezer at -20°C.

All samples of bacterial DNA were tested for quality. The 16S V3-V4 region of the sample was amplified by PCR. The 16S rRNA v3-v4 specific primers (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') were selected for PCR amplification. Then, the sample library was mixed quantitatively and the library was sequenced by Illumina MiSeq system (MiSeq,Illumina, San Diego, CA, USA).

Statistical analysis

The measurement data of the control, EB, and C-EB groups were compared using Student's test. Counting data were tested by a chi-square test and Fisher's exact probability method. SPSS 22.0 software (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis of the clinical data. The Shannon, Simpson, Goods Coverage, Observed Species, and Chao1 indices curves were drawn to evaluate the depth of sample sequencing and the diversity of intestinal flora (α - diversity) using Qiime process software(Qiime version 1,University of Colorado, Colorado, USA) and R language was used to draw the graph. For the operation classification unit (OTU) table, Qiime was used to generate a species distribution map at phylum level, and R language was used to draw the map. The Wilcoxon rank sum test was used to analyze the significant differences among the three groups of samples and the P value was calculated. Anosim (company, city, state, country) was used to test the differences between and within groups. A $P < 0.05$ has statistical significance.

Results

Clinical data

The basic clinical data of pregnant women and newborns included in this study are shown in Table 1. There were no significant differences in age, gestational age, mode of delivery, birth weight, and birth length between the three groups ($P > 0.05$). There were no significant differences in the male-to-female ratio between the three groups ($P > 0.05$).

The 16S rRNA sequencing analysis

All samples were sequenced to produce 1,267,914 effective sequences and 1,222,131 high quality sequences were generated after optimization. A total of 69,222 OTUs were obtained by classifying all sequences with 97% similarity. Among the sequences, there were 21 at the phylum, 37 at the class, 71 at the order, 138 at the family, and 297 at the genus levels. Each sample contained an average of 35,945 high-quality sequences and 2036 OTUs. The dilution curves for Goods Coverage (Figure. 2) and Observed Species (Figure. 3) were drawn. All the sequenced samples reached the platform stage, thus indicating that the sequencing quantity and depth were adequate to cover most of the bacteria.

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There was no significant differences in the Shannon, Simpson, and Chao 1 indices among the three groups ($P > 0.05$; Figures. 4-6), indicating that there was no significant difference in α diversity among the three groups; however, based on the Shannon and Simpson index charts, it was apparent that the Shannon and Simpson indices of the control group were higher than the EB and C-EB groups. Therefore, the species richness and evenness of the control group were higher than the other two groups.

Community composition of intestinal flora

At the phylum level, the community structure of intestinal flora in the three groups was similar (Figure 7). Proteobacteria, Firmicutes, and Actinobacteria are the three major phyla In each sample. The abundance of Proteobacteria and Firmicutes was higher than Actinobacteria.The abundance of Fusobacteria in the C-EB and EB groups was significantly lower than the control group.

Difference analysis of intestinal flora

At the genus level, the abundance of *E. coli* /*Shigella* and*Enterobacteriaceae* in the control group was the highest, while*Staphylococcus* and *Streptococcus* in the EB group was the highest. The most abundant bacteria in the C-EB group were*Enterobacteriaceae* and *E. coli* / *Shigella* . The abundance of E. coli/Shigella in the EB and C-EB groups was significantly lower than the control group ($P < 0.05$). while the abundance of *Staphylococcus* , *Methylobactrium* , and *Curvibacter* in the EB group was significantly higher than the other groups (Figure 8).

Anosim analysis

According to Anosim analysis, the difference among the three groups was greater than the difference within the group (r = 0.211) and the difference between the three groups was significant ($P = 0.001$ and $P < 0.05$; Figure 9).

Discussion

Main findings

There was no significant difference in the intestinal flora diversity and community structure between the FEB and the normal fetus. At the phylum and genus level,There were differences in the abundance of intestinal flora among the three groups.The microbiota may be related to the formation of echogenic bowel.

Strengths and limitations

At present, the mechanism of FEB is still unclear. This is the first study with a focus on the relationship between FEB and intestinal microbiota. The limitation of this study is that the sample size is small and the correlation analysis of fetal chromosome, meconium composition is not done。

Interpretation

In 1985, Lince[20] first reported that the FEB is an abnormal ultrasound sign. Slotnick[5] classified the strong echo of the intestine into three grades. In this study, all the cases were intestinal hyperechoic grade > 2.

In recent years, the intestinal flora play an important role in a number of diseases, such as premature delivery, obesity.[21]Therefore,we hypothesized that hyperechogenic bowel might be related to intestinal flora.

The study showed that there were abundant bacteria in the meconium of newborns, which is consistent with the results of Jimenez et al.[11]A reduction of the α diversity of the intestinal flora is causal of many diseases, including autoimmune diseases, obesity.[22-23] In this study there were no significant differences in the α diversity of meconium flora among the three groups and the composition of intestinal flora in the three groups was similar, which may be one of the reasons for the good outcome of most newborns with FEB. Pately et al.[24] reported that if the fetus with echogenic bowel can be delivered normally, there are no severe pathologic changes in the intestinal tract after delivery, which may be explained by our experimental results. In this study the structure of intestinal microbiota of the three groups of newborns was similar, with*Proteobacteria* and *Firmicutes* the two major components of the phylum, which is in agreement with Collado et al.[10]

What are the acoustic physical reasons for an echogenic bowel? Some researchers believe that the components in the lumen of the intestine are responsible for the formation of echogenic bowel, such as meconium. During the second trimester of pregnancy, echogenic bowel may be caused by the concentration and viscosity of the intestinal secretions. With the gradual release of these concentrated substances into the intestine, the grade of FEB will also decrease.[5]

IUGR is the main cause of perinatal death and morbidity. IUGR leads to the delayed development of colonic mucosa and the destruction of intestinal mucosal barrier, including intestinal epithelium, mucous layer, and other components.[25] Mesenteric ischemia and impaired intestinal motility are the result of fetal blood redistribution in newborns with IUGR, thus there is a preferential supply of blood flow to the heart and brain and reduced blood flow to other organs of the fetus, including the intestine, while FEB is one of the features representing IUGR.[26] Sepulveda et al.[27] considered that FEB in newborns with IUGR indicates that intestinal peristalsis slows down and meconium is thick due to intestinal ischemia.

CF is an autosomal recessive genetic disease. Because of the incomplete function of the CFTR gene, the airway mucus secretion increases and the mucous membranes thicken, leading to chronic lung infections.[28] The prenatal diagnosis of fetuses with FEB requires further consultation with parents whether or not the fetuses have CF because FEB is closely related to the CF pathologic state.[29] Thus, the appearance of FEB may also be due to the viscous secretion of mucus. Ultrasound reveals scattering and shows strong echoes of the fetal intestine.

Therefore, we found that the formation of FEB may be related to the secretion of mucus in the intestinal tract. The difference in bacterial abundance in the intestinal tract may promote or inhibit the secretion of mucus. Thus, different concentrations of mucus contributed to intestinal echoes. In the fetal small intestine, mucus is secreted as early as the 10th week gestation. Mucus secretion is helpful for the formation of meconium.[30] The surface of the fetal intestinal epithelium is covered with a thick layer of mucus, forming a hydrophilic network structure.[30-32] The mucus layer is secreted by goblet cells of the intestinal epithelium.[33] Mucus also includes water, ions, and immune mediators, which help to eliminate intestinal pathogens[34-35] and maintain the homeostasis of host microorganisms.[36] There were significant differences in the abundance of *E. coli / Shigella* ,*Staphylococcus* , *Methylrod,* and *Curvibacter* among the three groups of newborns, which may have resulted in different concentrations of mucus in the intestine and different intestinal echogenicity. With time, the abundance of bacteria in the intestinal tract changes, the mucus is diluted, and the echo is reduced. Therefore, part of FEB gradually disappears as gestational age increases. Therefore, we speculate that the difference in the abundance of intestinal flora might be related to echogenic bowel.

Caspi et al.[8] believe that the bacteria propagate, ferment, and metabolize in the intestine to produce intestinal gas, which is characterized by echogenic bowel after the fetus swallows amniotic fluid. Therefore, we propose that the formation of FEB may be related to the accumulation of intestinal gas, which may be related to intestinal bacteria. In the current study, a different abundance of*Gammaproteobacteria* , *Fusobacteria* ,*Enterobacteriaceae* , and *E. coli* were detected in the EB and C-EB groups.

Do these bacteria produce gas that makes hyperechogenic bowel? Some*Enterobacteriaceae* strains can produce amines.[37] Barton et al.[38-39] found that many bacteria can decompose cysteine into H_2S gas in the colon, including *Clostridium* , *Enterobacter* ,*E. coli* , and *Vibrio desulfuricus* . This suggest that the reason for the hypergenic bowel may be related to the gas produced by the microbiota. The accumulation of intestinal gas and multiple interfaces lead to FEB. Ljssennagger et al.[40] proposed that H_2S gas interacts with the intestinal mucous layer to regulate intestinal inflammation. We speculate that the intestinal microbiota might affect the secretion of intestinal mucus and intestinal gas to make a strong intestinal echo.

In this study the abundance of *Firmicutes* in the EB group was higher than the C-EB and control groups, while the abandance of Fusobacteria in the C-EB and EB groups was significantly lower than the control group. Indiani et al.[41] showed that the abundance of *Firmicutes* was positively correlated with the increase in the weight of children. The higher the abundance of*Firmicutes* , the higher the risk of obesity in children. This finding suggests the EB group has a higher risk of obesity in childhood.*Fusobacteria* is related to premature delivery, adverse pregnancy outcomes, and intra-amniotic infections.[42] In this study the abundance of Fusobacteria in the C-EB and EB groups was significantly lower than the control group. Therefore, we cannot give a reasonable explanation. At the genus level, the abundance of *E. coli / Shigella* in the EB and C-EB groups was significantly lower than the control group. *E. coli / Shigella* can cause watery diarrhea, dysentery, and other symptoms.[43] This means the possibility of diarrhea and other symptoms is lower in the EB and C-EB groups after delivery, which is an extremely interesting result. we do not know the specific

reason.

Conclusion:

The microbiota may be related to the formation of echogenic bowel via secretion of mucus and gas in the gut.

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Conflict of Interest: None declared.

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Ethical Statement: This study was approved by the ethics review committee of the Second Affiliated Hospital of Fujian Medical University on June 6, 2016 [Approval NO 2016.25].All patients signed written informed consent before participating in the study program.

Contribution to authorship

GL and YPZ were involved in conception and design of the study.Data analysis and interpretation were undertaken by LL , BZ,YTZ and YZ. YPZ and GL wrote the first draft and it was subsequently critically reviewed by all authors.All authors approved the final manuscript.

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