Developmental Patterns of Fecal Bile Acids in Healthy Neonates and Children

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCE 1 **Jing-Jing Xiong**
BCF 1 **Hong-Wei Hu**
C 2 **Chuan-Zhi Xu**
BC 3 **Jian-Wen Yin**
B 1 **Mei Liu**
B 1 **Li-Zhi Zhang**
B 4 **Yong Duan**
ABCE 1 **Yong-Kun Huang**

1 Department of Pediatrics, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, P.R. China
2 Department of Statistics, School of Public Health, Kunming Medical University, Kunming, Yunnan, P.R. China
3 Yunnan Center for Disease Control and Prevention, Kunming, Yunnan, P.R. China
4 Department of Clinical Laboratory Medicine, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, P.R. China

Corresponding Author: Yong-Kun Huang, e-mail: 13577097854@163.com

Background: Normal profiles of FBAs in healthy neonates and children in Kunming city and surrounding areas in China have not been previously determined. The objective of this study was to determine a developmental pattern of fecal bile acids (FBAs) in healthy neonates and children.

Material/Methods: A cross-sectional study was performed on 238 healthy neonates and children recruited in the First Affiliated Hospital of Kunming Medical University, China from October 2015 to September 2016. Secreted primary and secondary FBAs in fresh feces were quantitated by liquid chromatography mass spectrometry (LC-MS). Amounts of FBAs in feces were compared among various age groups.

Results: Trace amounts of cholic acid and chenodiol acid of primary FBAs were detectable at day 3 after birth, with a significant increase from day 3 to day 7. The primary FBAs gradually decreased from day 25 to the age of 6 years old. In contrast, a significant amount of glycochenodeoxycholic acid was detected on day 3 but decreased to a trace amount by day 7 and onwards. Primary FBAs appeared to maintain a high level, accounting for 98% of total FBAs, with no significant changes from day 7 to day 25 after birth. They gradually decreased from 90% to 10% from age 6 months to 6 years old. While the secondary FBAs were barely detected in neonates, only accounting for 2% of total FBAs, they were gradually elevated to 90% of total FBAs from age 6 months to 6 years old.

Conclusions: The liver can effectively synthesize primary bile acids 7 days after birth, and fecal primary bile acids tend to be stable after the neonate stage. Secondary bile acids continuously increase along with the maturation of intestinal flora, which reaches a relatively stable level at around 3 years old.

Keywords: Bile Acids and Salts • Child • Cross-Sectional Studies • Liver

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Background

Bile acids (BAs) are primarily synthesized from cholesterol in the hepatocytes of the liver through a series of steps of sterol ring hydroxylation and side chain oxidation [1]. In humans, the primary BAs synthesized in the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA). After being secreted into the intestine, the primary bile acids are metabolized into secondary bile acids, deoxycholic acid (DCA), ursocholic acid (UCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA)) by the gut microbiota [2]. Both primary BAs and secondary BAs are conjugated with glycine and taurine and then released in the small intestine to facilitate lipid emulsification, digestion, and absorption. In physiological conditions, the conjugated BAs are decomposed in the upper ileum and colon by colonized bacteria and most of intestinal bile acids are reabsorbed at the terminal ileum for enterohepatic circulation [3,4].

Bile acids solubilize dietary fat and activate pancreatic colipase to lipase for the absorption of dietary lipids, excrete cholesterol, and have an antimicrobial effect [5]. In addition, BAs are also ligands for the farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (GPBAR1) [6]. A previous study found that the amount of total BAs in feces decreased between birth and 7 days of age and thereafter increased in the neonatal period. The amount of total BAs at 7 days of age was significantly lower than at 6 to 12 months or at 2 to 4 years [7]. The study indicated that the hepatic excretion and transport of BAs were immature in infants who were younger than 3 months of age. Another study found that the level of primary BAs in children's feces increased at 3 months of age and reached a peak at 6 months of age before decreasing at 12 months of age. The level of secondary BAs started increasing at 6 months of age and dramatically elevated between 6-12 months of age before reaching a peak at 24 months of age. A decrease in level of primary BAs and an increase in secondary BAs coincided with weaning [8]. The study suggested that the change in gut microbiota from infants to adults after weaning and the diet might contribute to the profile of BAs in children.

Neonates and children undergo significant diet changes during development. In addition, the maturation of intestinal flora is an extensive process for many years in early life. It has been suggested that the maturation of liver function and the evolution of intestinal flora during early life stages can significantly affect the excretion of FBAs [9-11]. A significant change in the secretion of fecal BAs in children may indicate the existence of liver and inflammatory bowel diseases, chronic diarrhea, and allergic purpura [12-19]. Therefore, it is critical to understand the profile and changes of FBAs in neonates and children. In this study, we performed a cross-sectional analysis and determined the normal profiling of FBAs in healthy neonates and children in Kunming city, China.

Material and Methods

Study Design

A cross-sectional study was designed to quantify the amount of FBAs in the developmental stages of neonates and children enrolled at the First Affiliated Hospital of Kunming Medical University, Kunming, China from October 2015 to September 2016. Healthy neonates who were born in the Department of Obstetrics and healthy children who underwent regular physical examination in the Department of Pediatrics were randomly sampled. The study was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University. Written consent forms were signed by the parents of neonates and children enrolled before the study began.

Study Population

The study included 2 populations. Neonates were enrolled based on the following inclusion criteria: 1) Born in the Department of Obstetrics of the First Affiliated Hospital of Kunming Medical University between October 2015 and September 2016; 2) Full-term natural birth; 3) Subjected to exclusive breastfeeding. Neonates whose mothers had abnormal results of physical and laboratory examinations or who were administered antibiotics before delivery were excluded. Children were enrolled if they met the following inclusion criteria: 1) age 6 months to 6 years old; 2) Body mass index (BMI) in normal range (18-23); 3) no allergic, gastrointestinal, or immune system diseases, and no family history of genetic and metabolic diseases. Children subjected to antibiotics or microecological agents within 4 weeks before screening were excluded.

Detection and Quantification of FBAs

Fresh fecal samples were collected and immediately stored at -80°C in a freezer. Before testing, samples were thawed at room temperature and pretreated as previously reported [20]. Briefly, BAs were extracted with 2 mL methanol per gram fecal sample. After vertexing, the mixture was centrifuged for 5 min at 4000 rpm and the supernatant was filtered through a 0.22-micron filter. An aliquot (10 ul) of the extracted sample was loaded onto ZORBAX RRHD 2.1×50×18 micron chromatographic column (Agilent Company, USA) on the 1290 ultra-high performance liquid chromatography (Agilent Company, USA) equipped with the API4000 triple quadrupole mass spectrometer (AB Company, USA). A mixture of 0.1% formic acid and acetonitrile was used as the eluent, and the separation was carried out by step gradient elution at a flow rate of 0.3 ml/min. The mobile phase composition was gradually changed as follows: formic acid-acetonitrile (95: 5, v/v) for 0-5 min, formic acid-acetonitrile (4: 6, v/v) for 5-15 min, formic acid-acetonitrile (5: 95, v/v) for 15-17 min, and formic acid-acetonitrile,

Table 1. Changes of primary FBAs in neonates at different days after birth.

Age (days)	Subject (No)	CA (pg/g of feces)		CDCA (pg/g of feces)		GCDCA (pg/g of feces)	
3	22	0.928	[0.2969, 52.0052]	0.1690	[0.0679, 13.3778]	3.9738	[1.5030, 13.0059]
7	22	103.2204	[50.8798, 131.0697]*	16.2087	[7.9644, 39.3262]*	0.1900	[0.0523, 0.8065]*
14	20	48.2144	[30.9034, 73.4583]*#	17.2035	[9.7934, 27.5860]*	0.1383	[0.0452, 0.5895]*
25	22	54.8408	[40.6006, 70.7017]*	30.3179	[13.6998, 42.8812]*	0.2737	[0.0717, 1.6385]*

* P≤0.01, compared to day 3; # P≤0.01, compared to day 7.

Table 2. Changes of primary FBAs in children at different ages.

Age (m or y)	Case (N)	CA (pg/g of feces)		CDCA (pg/g of feces)		GCDCA (pg/g of feces)	
6 m	30	82.1533	[55.0064, 128.6705]	43.6521	[14.6040, 69.6985]	0.3914	[0.2036, 1.8131]
1 y	30	54.4660	[31.7422, 90.2598]	30.2234	[10.2402, 49.6632]	0.6614	[0.3838, 1.4999]
2 y	30	64.4587	[18.5599, 90.7932]	34.9018	[5.7256, 63.2624]	1.5227	[0.3930, 4.6325]
3 y	30	64.3077	[1.7156, 225.7426]	78.8073	[6.5687, 188.1188]	0.8986	[0.5268, 1.4977]
6 y	30	14.0255	[1.3673, 120.5654]	21.9598	[5.9020, 110.9196]	1.2544	[0.5470, 2.2862]

95: 5, v/v for 2 min. For the MS detection, the voltages were set as follows: 1) port voltage was -10 V, 2) collision voltages were set as -77, -30, -30, -30, -30 and -40 V, 3) outlet voltages of the collision chamber were -11, -20, -20, -20, -20 and -20 V.

Standards (Canadian TRC, Toronto Research Chemicals, Toronto, Canada) were used to estimate the retention time and mass charge ratio of FBAs. The retention times of glycochenodeoxycholic acid (GCDCA), ursodeoxycholic acid (UDCA), chenodiol acid (CDCA), deoxycholic acid (DCA, cholic acid (CA), and lithocholic acid (LCA), were 13.41, 11.71, 13.04, 13.30, 12.00, and 14.57 min, respectively, and the m/z GCDCA, UDCA, CDCA, DCAm CA, and LCA were 448.3, 391.2, 391.2, 391.2, 407.2, and 375.3, respectively.

Statistical Analysis

SPSS 19 (IBM, Armonk, NY, USA) was used for statistical analyses. The normally distributed data were expressed as mean and standard deviation (x±s). Comparisons among multiple groups were performed using one-way ANOVA with SNK-q for post hoc tests. The skewedly distributed data were expressed as median [1st quartile, 3rd quartile]. Kruskal-Wallis analysis was used to test the difference among groups. Mann-Whitney test was used to determine the difference between 2 groups. The correlation between age and BA was analyzed by Spearman rank correlation test. P<0.05 was considered as statistically significant.

Results

FBA concentrations were quantified in neonates at day 3, 7, 14, and 25 after birth. As shown in **Table 1**, the levels of primary BA, CA, and CDCA were barely detected at day 3. However, they were dramatically increased by more than 100 times at day 7 and reached 103 and 16 pg/g, respectively. While the amount of CDCA was continually increased at day 14 and day 25, the level of CA was decreased at day 14 and day 25. In contrast, the amount of GCDCA reached approximately 4 pg/g at day 3 and then decreased at days 7, 14, and 25. We found that the changes in FBAs were significantly different at day 14 as compared to day 7. Our Spearman analyses indicated that there were significant positive correlations of CA and CDCA with age (r=0.224 and r=0.426, respectively, both $P<0.05$), but there was a negative correlation of GCDCA with age (r=-0.379, $P<0.05$). There were no significant changes in the levels of primary FBAs among children at ages of 6 moths to 6 years (**Table 2**). In contrast to the primary FBAs, the amounts of secondary FBAs in neonates were very low (**Table 3**). The amounts of DCA and LCA in neonates' feces were very low too, but were detectable at ages 3, 7, 14, and 25 days. There was a decrease in DCA but an increase in LCA at day 7 as compared to day 3. There were no significant changes in UDCA among age groups of neonates after birth. However, our Spearman analysis showed a significant negative correlation of DCA with age (r=-0.480, $P<0.05$) and a positive correlation of UDCA with age (r=-0.295, $P<0.05$). There was no correlation of LCA with age (r=0.195,
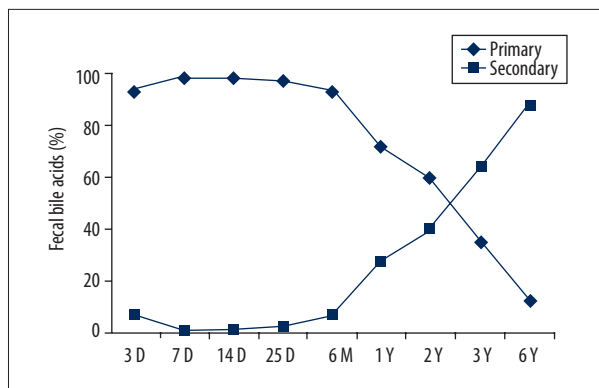
Figure 1. Changes of primary and secondary FBAs in neonates and children.

P>0.05). The amount of total BAs in feces was increased by 26-fold from day 3 to day 7 (4.5 vs 120 pg/g) (**Figure 1, Table 3**).

There was a significant increase in secondary FBAs in children aged 6 months to 6 years (**Table 4**). The amount of DCA was gradually elevated by approximately 4000-fold from age 6 months to 6 years old. The levels of LCA were also increased by 3884-fold from age 6 months to 3-6 years old, while the amount of UDCA was elevated by approximately 6-fold from age 6 months old to 3-6 years old. Spearman analysis showed positive correlations of all 3 FBAs – DCA, LCA, and UDCA – with children's age (r=0.757 for DCA, r=0.724 for LCA, and r=0.357 for UDCA; all *P*<0.05).

The mean percentage of primary FBAs was increased from 93% of total FBAs to 99% from birth to 7 days old, then gradually decreased to 10% at age 6 years old. In contrast, the percentage of secondary FBAs at 7 days of age decreased from 7% of total FBAs to 1% from birth to 7 days old and then gradually increased to 90% of total FBAs at age 6 years old (**Figure 1**).

Discussion

In this study, we demonstrated that primary FBAs, CD, and CDCA started to be detectable and were significantly increased from day 3 to day 7 after birth in neonates. The amounts of total BAs in feces increased by 26-fold from day 3 to day 7. The primary FBAs gradually decreased from day 25 to age 6 years old. In contrast, a significant amount of GCDCA was detected at day 3 but decreased to a trace amount at day 7 and afterwards. Primary FBAs appeared to maintain a high level, accounting for 98% of total FBAs, with no significant changes from day 7 to day 25 after birth. They were gradually decreased from 90% to 10% from 6 months to 6 years old. While the secondary FBAs were barely detected in neonates, only accounting for 2% of total FBAs, children's secondary FBAs were gradually elevated to 90% of total FBAs from 6 months to 6 years old. Our study results agree with previous findings, suggesting the liver maturation at this stage supports initiation of effective primary BA synthesis [21]. At the same time

Table 3. Changes of secondary FBAs in neonates at different days after birth.

Age (days)	Case (N)	DCA (pg/g of feces)		LCA (pg/g of feces)		UDCA (pg/g of feces)	
3	22	0.0646	[0.0384, 0.1382]	0.0050	[0.0024, 0.0130]	0.2689	[0.1083, 0.9913]
7	22	0.0107	[0.0076, 0.0166]*	0.0132	[0.0104, 0.0236]*	0.6618	[0.3849, 2.0035]
14	20	0.0371	[0.0189, 0.0532]*#	0.0142	[0.0096, 0.0254]*	0.6861	[0.1602, 2.0837]
25	22	0.0089	[0.0041, 0.0230]*@	0.0129	[0.0069, 0.0243]*	2.1994	[0.2620, 6.9686]

* P≤0.01, compared to day 3; # P≤0.01, compared to day 7; @ P≤0.01, compared to day 14.

Table 4. Changes of secondary FBAs in children at different ages.

Age (m or y)	Case (N)	DCA (pg/g of feces)		LCA (pg/g of feces)		UDCA (pg/g of feces)	
6 m	30	0.0344	[0.0146,0.0667]	0.0262	[0.0167,0.2720]	8.7172	[2.1052,30.0651]
1 y	30	0.0761	[0.0368,3.1575]*	0.1157	[0.0482,1.4295]	32.2537	[17.4224,53.6974]*
2 y	30	18.7148	[0.1404,53.2972]*#	6.8367	[0.7488,50.0448]*#	42.0231	[18.7474,60.8055]*
3 y	30	110.5023	[15.5455,230.3318]*#@	100.9901	[2.1096,318.5185]*#@	55.2294	[27.9412,182.4074]*#
6 y	30	134.9758	[71.1080,189.3100]*#@	83.1608	[43.8175,209.1294]*#@	48.8465	[11.6218,123.3991]*

* P≤0.01, compared to 6-month old; # P≤0.01, compared to 1-year old; @ P≤0.01, compared to 2-year old.

period, GCDCA, the product of conjugation of primary BA and glycine in the liver, remained low in feces of neonates and significantly declined after 7 days of birth, which may be caused by the digestion of GCDCA due to initiated colonization of intestine flora. It has been pointed out that the abundance and variety of fecal flora, including the abundance of Bacteroides, increases with time after birth [22-25], and that Bacteroides in feces can catalyze decomposition of bile acids [26-28]. Our data on total FBAs in neonates disagree with Kimura's report, in which the amounts of total BAs in feces decreased between birth and 7 days of age [7]. This difference could be explained by the following different quantification approaches: 1) In this study, we only included CA CDCA and GCDCA of primary BAs and DCA, LCA, and UDCA of secondary BAs. We did not quantify the amount of sulfated BA, which accounts for 54% of total FBAs after birth [7]. 2) We did not completely recover the excreted BAs after solvolysis, hydrolysis, and derivatization. 3) We only took the samples on day 3 and 7. We did not take neonate's samples at day 0 and had no data of the level of total FBAs at day 0. In children aged 6 months to 6 year, their primary FBAs were at similar level as those in neonates after 7 days and no significant difference was detected among different age groups of children. Our results indicate that after the maturation of liver function and very early colocalization of microbiota, the primary FBAs tend to stay stable.

The secondary BAs in feces were mainly DCA and LCA. We found that although trace amounts of secondary FBAs could be detected and were statistically significant in neonates, the general levels of secondary FBAs were very low. Our results suggested that the intestine biota was the major source of fecal secondary BAs. It has been reported that Clostridia can catalyze the conversion of primary BA 7α-hydroxydeoxygenation to secondary Bas [26-28]. Since the abundance and diversity of intestinal flora were both low at the early stage of the neonates and the *Bacteroides monomorphus, Bifidobacterium longum, Bifidobacterium adolescentis, Bacteroides ovalis, Bacteroides dorei,* and *Escherichia coli* became the dominant strains [22,25], the efficiencies of intestinal flora to convert primary BAs into secondary BAs remained at low levels. Other studies have shown that the intestinal flora keep maturing during childhood, and that 2 to 3 years after birth is the key period for the establishment of an intestinal flora with the absolute dominance of anaerobic bacteria, which gradually change to adult-like flora at age 4 to 5 years [29-31]. In this study, we observed continuous increase of secondary FBAs in children aged 6 months to 6 years. A significantly accelerated increase after age of 2 was

detected, which tended to get more stable at age 3 to 6 years. These results are consistent with the changes in intestine flora previously reported [39-31], indicating that the secondary FBAs were mainly generated by intestinal flora.

Although we performed a cross-sectional study in 238 healthy neonates and children in the First Affiliated Hospital of Kunming Medical University, China and provided a developmental pattern of fecal bile acids that could be used to determine the abnormal levels of FBAs associated with metabolic diseases, there were certain limitations in this study. First, the sample size was not big enough to estimate a normal range of FBAs in neonates and children, but we could detect relative trends of changes. Second, the time points assessed were not enough to illustrate the detailed variations of FBAs, although a general trend of change could be observed. More time points are needed in future studies. Third, we only evaluated that levels of FBAs and did not consider numerous factors such as the liver function and composition intestinal flora that could affect the levels of FBAs in children. Fourth, this study only provides information about several qualitative changes with age in neonates and children and lacked data on the quantitative fecal excretion of bile acids that reflects BA synthesis. In future research, GC-MS analysis should be used to obtain a complete recovery of BA excretion after solvolysis, hydrolysis, and derivatization. It is also critical to evaluate the level of sulfated BA in FBAs.

Conclusions

In summary, we performed a cross-sectional study in healthy neonates and children in the First Affiliated Hospital of Kunming Medical University, China and found a developmental pattern of fecal bile acids that could be used determine abnormal levels of FBAs associated with metabolic diseases. We found that the liver could effectively synthesize primary BAs 7 days after birth, and fecal primary bile acids tend to be stable after the neonate stage. While the fecal secondary BAs continuously increased along with the maturation of intestinal flora, they increased until the age of 3 years old and then remained stable. There was an increase in DCA but a decrease in both LCA and UDCA.

Conflict of Interest

None.

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