Early colonization of intestinal bifidobacteria and lactobacilli in the postoperative neonates with congenital intestinal atresia

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ABSTRACT

الأهداف: مراقبة الاستعمار المعوي المبكر لبكتيريا البيفيدو، وبكتيريا اللاكتوباسيلين وذلك بعد إجراء العملية الجراحية للأطفال حديثي الولادة إثر إصابتهم برتق الأمعاء الخلقي.

الطريقة: أجريت هذه الدراسة الاستطلاعية في مستشفى شانغهاي للأطفال، شانغهاي، الصين وذلك خلال الفترة من فبراير 2009م إلى أغسطس 2010م، وفيها تم تقسيم المشاركين إلى مجموعتين: مجموعة الأطفال حديثي الولادة والمصابين برتق الأمعاء الخلقي وكان عددهم 18 طفلاً، ومجموعة الأطفال السليمين الذين أكملوا طور الحمل ويرضعون من حليب الأم الطبيعي وقد كان عددهم 20 طفلاً. لقد تم الكشف عن أعداد البكتيريا كلاً على حدة وذلك من خلال اختبار عينات البراز بواسطة استخدام الوقت الفعلي من التفاعل التسلسلي المبلمر والفلوروسين وذلك على مدار 6 مراحل مختلفة من الوقت.

النتائج: أشارت نتائج الدراسة إلى انخفاض متوسط مستويات بكتيريا البيفيدو واللاكتوباسيلين (لوغاريتم/غرام من البراز) في عينات مجموعة الأطفال حديثي الولادة والمصابين برتق الأمعاء الخلقي (ج1) بالمقارنة مع مجموعة الأطفال السليمين (ج2) [البيفيدو :6.3 في ج1 مقابل 9.9 في ج2]، [اللاكتوباسيلين6.9: في ج1 مقابل 7.6 في ج2]. ولقد ظهر اختلافاً واضحاً بين المجموعتين عند تقدير الوقت اللازم بالأيام لاستعمار نوعي البكتيريا [البيفيدو :5.8 في ج1 مقابل 3.0 في ج2]، [اللاكتوباسيلين:4.2 في ج1 مقابل 1.2 في ج2]. لقد كان الاستعمار المعوي لبكتيريا اللاكتوباسيلين أسرع من بكتيريا البيفيدو وذلك في كلي المجموعتين، كما وقلت مستويات البيفيدو باستمرار في ج1 مقارنةً بمستويات اللاكتوباسيلين وذلك عكس ما حدث في ج2.

خاتمة: أثبتت الدراسة مدى تأثر الاستعمار المعوي لبكتيريا البيفيدو واللاكتوباسيلين في مجموعة الأطفال حديثي الولادة وذلك إثر إصابتهم برتق الأمعاء الخلقي، ولقد كانت مستويات تكاثر بكتيريا البيفيدو أعلى من بكتيريا اللاكتوباسيلين.

Objectives: To observe the early colonization of bifidobacteria and lactobacilli (B&L) in the postoperative neonate patients (NPs) with congenital intestinal atresia (CIA).

Methods: A prospective study was conducted in Shanghai Children's Hospital, Shanghai, China between February 2009 to August 2010 on 18 postoperative NPs with CIA (NP group), and 20 healthy full-term neonates raised by breastfeeding (healthy group). The fecal B&L in the 2 groups of neonates were consecutively quantified by real-time fluorescence quantitative polymerase chain reaction on 6 different time points.

Results: The mean levels (log/g feces) of B&L in the NPs group were significantly lower than in the healthy group at the end point of study (bifidobacteria: NPs [6.3] versus healthy [9.9]; lactobacilli: NPs [6.9] versus healthy [7.6]). Significant differences between the 2 groups also existed on the colonization time (days) of the intestinal B&L (bifidobacteria: NPs [5.8] versus healthy [3.0]; lactobacilli: NPs [4.2] versus healthy [1.2]). Both groups colonized lactobacilli earlier than bifidobacteria. During the study period, levels of bifidobacteria in the NPs group were continuously decreasing compared to lactobacilli, which was opposite to the healthy group.

Conclusion: The colonization of the intestinal B&L in the postoperative NPs with CIA was severely interfered. The proliferation of bifidobacterium was more restrained than lactobacillus.

Saudi Med J 2011; Vol. 32 (3): 265-270

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Received 26th October 2010. Accepted 17th January 2011.

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The establishment of gut microbiota should not be regarded as a succession in the ecological sense, but rather as a complex process influenced by interactions between the microbial and the host, and by external and internal factors.[1] Of the gut flora, the lactic acid bacteria such as bifidobacterium and lactobacillus (B&L) are the important physiologic probiotics.[2] The secretion and metabolites of these physiologic probiotics are closely related to archenteric maturation process of the neonate, which could not only promote the growth and proliferation and functional differentiation of intestinal epithelial cells,[3] but can also give effect on immune activation.[4] Therefore, their quantities reflect the intestinal physical condition up to some extend.[5] Congenital intestinal atresia (CIA) is more common on gastrointestinal malformation of the neonate, which is the primary cause of intestinal obstruction in neonate.[6] Its characteristic is stagnant growth of intestine with a single or multiple intestinal atresia (IA). Surgical operation is the only effective treatment to recover continuous and unimpeded intestinal canal. Postoperative survival rate of CIA was high (>90%)[7] due to the improvement of operation techniques, care in perioperative period, and clinical application of parenteral nutrition. As survival rate is enhancing, the objective of further treatment of neonatal patients (NPs) with CIA after structure reconstruction is how to recover intestinal function. The disorders of intestinal motility in the NPs with CIA for a long time have been observed by several researchers.[8,9] However, the intestinal flora changes of the postoperative NPs with CIA, especially the early colonization of B&L, has not yet reported. The aim of this study is to observe the early colonization of B&L in the postoperative NPs with CIA, using the real-time quantitative polymerase chain reaction (PCR) method.[10]

Methods. This prospective study was conducted between February 2009 and August 2010 at Shanghai Children's Hospital, Shanghai, China. The study was approved by the Joint Committee of Ethics of Shanghai Children's Hospital, Shanghai, China. Eighteen NPs with CIA (NP group) and 20 breastfed healthy, full-term infants (healthy group) comprised the study population. Neonatal patients group were studied from the time of admission from the first day of life, until the fourteenth postoperative day (**Table 1**). Informed consent was obtained from guardians of the neonates. Neonatal patients with abnormalities of the gastrointestinal tract were included in the study. All NPs had non-defecation before operation. They undergo operation within 12 hours of admission (from 4-12 hours). Healthy full term neonate was defined as follows: gestational age ≥37 weeks, birth weight ≥2500 g, Apgar score at 5 min ≥8, no history of chronic disease and acute infectious disease in the mother; and no defect or fever in the infant during the study.[10] The neonatal patients who had malformations of other system except for alimentary tract, or had defecation before operation, or received a surgery more than 12 hours upon admission were excluded in the study.

Disclosure. This study was supported in part by the National Natural Science Foundation of China (No. 30801205), and supported in part by the Shanghai Science and Technology Committee (Project "Venus", No. 09QA1404800).

The fecal B&L of the NPs were consecutively quantified by real-time quantitative PCR on 6 postoperative days (day 1, 3, 5, 7, 10, 14). Of healthy neonates, we quantified them on day 1, 3, 5, 7, 10, 14 from birth. The nutritional support mode of all 18 NPs switched from parenteral nutrition (PN) to enteral nutrition (EN), and then was switched to total oral feeding. The antibiotic regimen was third-generation cephalosporins (Rocephin) combining with metronidazole.

Specimens and DNA extraction. The flora of the rectum was used as sample into a sepsis centrifugal tube with swabs, stored at -20°C, and extracted genome's DNA of fecal bacteria within 6 hours. The storage temperature of the DNA solution is -80°C before the quantitative analysis. Bacterial DNA from fecal samples was extracted using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany) with some modifications.[10]

Real time fluorescence quantitative PCR. The genus-specific 16S rRNA-targeted primers sets used for quantitative real-time PCR in this study were referred to the previous study.[10] The PCRs were performed in 20 µL final volumes in capillary tubes in a ABI Prism 7300 (Applied Biosystems, USA). Reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 200 µM concentration of each deoxynucleoside triphosphate, 0.5 µM each primer, 1:30,000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.), and 0.025 U of Taq DNA polymerase (Promega, Shanghai) per µl, and 1 µl of bacterial template DNA. All capillaries were sealed, centrifuged at 500 g for 5 seconds, and then amplified in an ABI- 7300 instrument. The amplification program for B&L was as follows: 95°C for 5 minutes, 40 cycles of 10 seconds at 95°C, 31 seconds at 61°C, and 30 seconds at 72°C,[10] comprised of activation of polymerase. The temperature transition rate was 20°C per seconds for all steps. The PCR product of bifidobacterium or lactobacillus was measured based on the similar amplification efficiency

among the amplification curves of standards and a sample.[10] Melting curves were used to determine the specificity of the PCR.[11] Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 95^{0}C at 60 seconds 55^{0}C at 60 seconds, and 55^{0}C-98^{0}C (10 seconds/cycle; 0.5^{0}C/cycle) 86 cycles. A specific product with a sharp, narrow peak was easily identified from the non-specific amplifications with a much lower melting temperature and a broader range by the melt curve analysis.[10] The melting curves revealed no presence of primer-dimers. The standard curve profiles of bifidobacterium and lactobacillus in this study was generated by the ABI software (Applied Biosystems, Foster City, CA, USA). Quantification of unknowns was achieved by using standard curves made from a standard dilution series of known concentrations of plasmid DNA containing an amplicon for each set of primers.[12] The resulting standard curve was shown as a graph of cycle number versus log concentration. For each sample, the crossing point was plotted against the known concentration of the standard. The crossing point (Cp) was defined as the second derivative maximum from the fluorescence curve. All calculated 'unknown' sample values must fall within the limits of the standards used to generate this curve. The detection limit of the real-time PCR procedure with the DNA extract from feces and the PCR condition described in this paper was found to be approximately 4x10^4 bacterial cells of bifidobacteria or lactobacilli per gram of feces (4.6 log units).

Statistical analysis. We used SPSS for Windows 13.0 statistical software for statistics. Numbers of B&L in feces were expressed using logarithm transformations of the data, which were normally distributed. Student's t-test was used for comparison of means. All tests were 2-tailed, and p-values of <0.05 were accepted as statistically significant. The data of colonization time and colonization level of B&L were presented as mean (minimum - maximum). The data development of B&L in the 2 groups were expressed in the statistical chart and was analyzed using SPSS.

Results. There was no difference between the 2 study groups with respect to birth weight, gestational age or gender ratio (**Table 2**). In the NPs group, none of the patients demonstrated signs of infections such as aspiration pneumonia, sepsis, and infection of incisional wound and so forth as well as complications, such as disruption of wound, anastomotic leakage, intestinal perforation, and postoperative intestinal obstruction. The average time to start the enteral feeding was 1.7 days (between 1 and 4 days); 7.2 days were needed to achieve the total enteral nutrition (TEN) average, and the 5.7 days antibiotic (from 3-11 days) was on average.

Quantification of bifidobacteria and lactobacilli. Generally, copy number of 16S rRNA genes of bifidobacteria or lactobacilli per grams of the collected fecal sample were quantified by using real time PCR and transformed into logarithms. On day 14, the mean levels of B&L in the NPs group were significantly lower compared with the healthy group. The mean levels of bifidobacteria were 6.3 in NPs group versus 9.9 in the healthy group. The mean levels of lactobacilli were 6.9 in NPs group versus 7.6 in the healthy group. Significant differences between the 2 groups also existed on the colonization time of the intestinal B&L, namely the time of the first positive quantitative analysis result of B&L in fecal specimen. The colonization time of the bifidobacteria was 5.8 days in NP group versus 3.0 days in the healthy group. The colonization time of the lactobacilli was 4.2 days in NP group versus 1.2 days in healthy group. The population and the levels of bifidobacterium and lactobacillus on day 14 between the 2 groups are summarized in **Table 2**. **Figure 1** showed the development of B&L in both groups. The colonization time of the intestinal bifidobacterium was always longer than lactobacillus in both groups. Moreover, the 2 groups had different increment speeds and levels of B&L. The high increment speed of bifidobacteria was appeared on day 3-5 in the healthy group while in the

Table 1 - Atresia parts and surgical management in 18 neonatal patients.

Atresia parts	No. of patients	Surgical management
Duodenum	4	Resection: Diamond-shaped side-to-side anastomosis
Jejunum	7	Resection: end-end anastomosis
Ileum	7	Resection: end-end anastomosis

Table 2 - The population and levels of bifidobacterium and lactobacillus on day 14 between the 2 groups.

Variables	NPs group	Healthy group	*P*-value*
Gestational age (%)	39.4	38.7	0.11
Birth weight (n)	3454	3532	0.43
Gender ratio			0.54
Male	9	12	
Female	9	8	
Bifidobacteria (log/g feces)	6.3 (5.2-8.3)	9.9 (7.9-10.9)	0.00
Lactobacilli (log/g feces)	6.9 (6.1-8.4)	7.6 (6.3-8.5)	0.01
Bifidobacteria (day)	5.8 (5-7)	3.0 (3-3)	0.00
Lactobacilli (day)	4.2 (3-7)	1.2 (1-3)	0.00

*As determined by the Independent-samples t-test.
NPs - neonatal patients

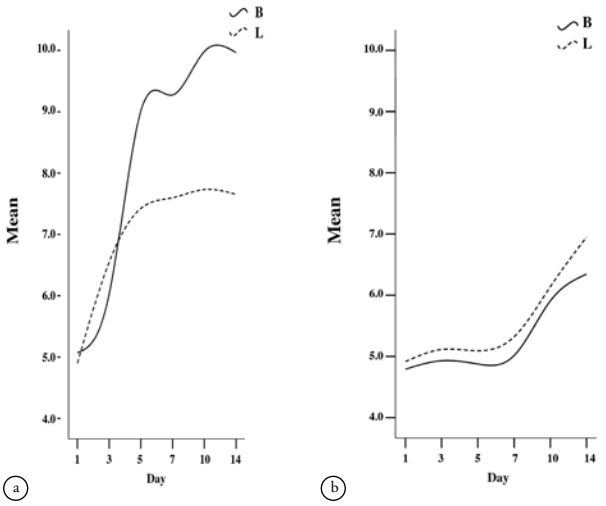

Figure 1 - Development of bifidobacterium (B) and lactobacillus (L) in the **a**) neonate patients group and **b**) healthy group

NPs group it appeared after a week. The high increment speed of lactobacilli appeared on day 1-3 in the healthy group while after 7 days in the NPs group. At the end point of the study, levels of B&L in the healthy group had reached a relatively stable state, and the levels of bifidobacteria were obviously higher than lactobacilli. During the study period, levels of B&L did not reach a high plateau and the levels of bifidobacteria were always lower than the lactobacilli in the NPs group.

Discussion. The microflora was formed in common evolutionary process between normal microbiota and its host. On the physiological status, they attached to the surface of intestinal mucosa to form the bio-membrane in order to prevent intrusion and colonization from pathogens, which is defined as colonization resistance.[13] Moreover, the multiplex vitamin and biological enzyme can be synthesized for the growth and development of the neonate. Autochthonous has flora antigenicity, which could stimulate immunologic function of neonates to establish.[4,14-16] The colonization of intestinal bacteria could be affected by many factors such as: mode of delivery, feeding patterns, premature, disease and antibiotic treatment. These factors could not only influence on the establishment of normal intestinal microcosmic ecological balance, but also the occurrence of infectious diseases during neonatal period.[2-5] The difference from the healthy group was that the intestinal B&L colonized in the first 1-3 days after birth; the colonization period of the intestinal B&L of the NPs with CIA obviously delayed and the colonization level also decreased (**Table 1** and **Figure 1**). It was concluded that the colonization of probiotics had been severely inhibited even though these neonates had a reconstruction of alimentary. This phenomenon may be related to many factors, such as intestinal disease, operation, antibiotic treatment, disuse of intestine including fasting and total parenteral nutrition (TPN); and so on. Thus, the intestine was in resting state during the postoperative fast and TPN. The intestinal microecosystem was hard to establish without the stimulation of food. On the other hand, this maybe related to the diseases, which there were some congenital defects in the intestinal structure and function. Several studies found that there were certain developmental defects, which handicapped the recovery of intestinal motility near the end and remote end of intestinal anastomosis; including neuronal dysplasia of intestinal wall, disproportion between the vertical and circle intestinal muscle, and the interstitial cytopathic effect, even though the digestive tract obstruction had been removed.[17-20] Hence, we speculated that the suffocation of recovery of intestinal motility deferred propulsion of food in the intestinal canal, then it was influenced by the energy supply requirement for bacterial colonization in the colon. Simultaneously, mucosal edema and bleeding caused by operation also hindered the normal adhesion of the lactic-acid bacteria to the intestinal mucosa. **Figure 1** showed that B&L in the NPs group had a relative rapid growth period after one week. Combining the average days of antibiotics usage (5.7 days) and achieving TEN (7.2 days), it seemed that withdrawing antibiotics and achieving TEN would help in the proliferation of intestinal B&L. This has been proved in some studies on neonates without CIA or animal experiments.[5,21-26] We also found that the levels of bifidobacteria were always lower than lactobacilli in the NPs group, and this was opposite to the healthy group and similar with the intestinal flora of the elderly.[10,14]

In view of the important role of bifidobacterium on growing development and immunity in neonates, the significance and long-term effects of this phenomenon on the NPs with CIA, which has never been reported before, merits further investigation. In addition, it was hard to conclude what should be responsible for this phenomenon. We speculated that the selection of the antibiotics and nutritional food might be some of the related factors. In this study, metronidazole, which all the NPs with CIA took for preventing abdominal infection, has the antibacterial action to most anaerobic bacteria, but aerobe and facultative anaerobe. Its antimicrobial spectrum includes *Bacteroides fragilis*, *Fusiform bacillus*, *Clostridium*, bifidobacteria, *Eubacterium*, *Coccus*, digestive coccus and so on. As a kind of obligate anaerobe, the proliferation of bifidobacterium maybe more obviously restrained than the facultative anaerobe such as lactobacillus by metronidazole. Moreover, non-breast feeding and enteral nutrient without bifidus factor

were both adverse to growth of intestinal bifidobacteria, which had been reported in many researches.[27-30] Our study showed that colonization levels of B&L of the postoperative NPs with CIA significantly decreased and there was an imbalance of the development of B&L. Rational use of antibiotics might be helpful in solving this problem in the early and rational use of intestine and breast-feeding Of course, some researchers[15,31-34] suggested that microbial ecological agents should be used in the early stage to assist the flora colonization of the neonates and to promote the establishment of intestinal microecology. In the future, whether it was safe to the postoperative neonates with incomplete intestinal barrier function, researches should perform this study, including animal experiments.

Limitations of this study were low incidence of CIA, no enough sample size (ascertainment bias may cause the interpretation of the data unclear). Potential limitation was unavoidable due to the NPs with different intestinal atresia parts that received different surgical management, which might influence the colonization of the normal flora. Finally, a 14-day follow-up period is not enough to observe the long-term effects of the disorder colonization in B&L on the growing development and immunological function of the NPs with CIA.

In conclusion, the colonization of the intestinal B&L of the postoperative NPs with CIA was severely interfered. The proliferation of bifidobacterium was more obviously restrained than lactobacillus.

Acknowledgment. *The authors would like to thank Mr. Xing Zhu, Department of Biostatistics, School of Medicine, Shanghai Jiaotong University for his help with the statistical analysis, and the nurses of the Department of Paediatric Surgery for their invaluable help in performing this study.*

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