Original Article
Bifidobacterium regulates TLR-4 and TLR-9 expressions in diarrhea rat caused by antibiotics

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Abstract: The incidence of diarrhea caused by antibiotics keeps rising following the application of antibiotics. Its occurrence induces intestinal flora disturbance and is closely related to immune dysfunction. This study intends to explore Toll-like receptor 4 (TLR-4) and TLR-9 expressions in diarrhea rat caused by antibiotics, and the regulatory role of Bifidobacterium treatment. SD rats were randomly divided into three groups, including control, diarrhea group established by lincomycin hydrochloride treatment, and Bifidobacterium group treated by Bifidobacterium intragastric administration upon diarrhea model. TLR-4 and TLR-9 mRNA expressions in mesenteric lymph nodes, intestine, and liver were detected by real-time PCR. Intestinal flora changes, and translocation amount of bacteria in mesenteric lymph nodes, intestine, and liver were analyzed by bacterial cultivation. NF-κB expression in intestinal tissue was determined by Western blot. TLR-4 and TLR-9 mRNA expression in mesenteric lymph nodes, intestine and liver was significantly downregulated, NF-κB level was obviously reduced, while intestinal flora disturbance and translocation appeared in diarrhea group compared with those in control (P < 0.05). TLR-4 and TLR-9 mRNA was upregulated, intestinal flora disturbance and translocation attenuated, and NF-κB was increased in Bifidobacterium group compared with those in diarrhea group (P < 0.05). Diarrhea caused by antibiotics inhibited TLR-4 and TLR-9 mRNA, induced intestinal flora disturbance and translocation, and downregulated NF-κB expression. Bifidobacterium promoted TLR-4 and TLR-9 expressions, improved innate immune, maintained intestinal flora balance, alleviated bacteria translocation, and upregulated NF-κB expression.

Keywords: Antibiotics, diarrhea, NF-κB, TLR-4, TLR-9, flora disturbance, bacteria translocation

Introduction

Diarrhea caused by antibiotics, also known as antibiotic-associated diarrhea (AAD), refers to diarrhea occurred after antibiotics application and associated with antibiotics. It is the most common form of iatrogenic diarrhea [1, 2]. Wide application of antibiotics and lack of understanding of antibiotics hazards causes serious unreasonable application of antibiotics [3]. Irrational use of antibiotics can not only lead to antimicrobial resistance, also induce AAD. AAD belongs to diarrhea following antibiotics application that cannot be explained by other reasons [4, 5]. Following the widely application of antibiotics, more than 700 kinds of medicines can cause diarrhea, while antibiotics account for more than 25% [6, 7]. Difference in immunity, race, and antibiotics types may cause diarrhea by different incidence [8]. Common irratio-

nal use of antibiotics in our country leads to the rising incidence of AAD. Thus, it is urgently needed to treat AAD [9]. AAD destroys normal intestinal flora, induces intestinal flora disturbance, and is closely related to immune dysfunction [10, 11].

Innate immunity, also known as natural immunity, is a series of defense mechanism formed in the process of long-term species evolution. Innate immunity responds to invading pathogens quickly and plays a nonspecific anti-infection effect. Moreover, it also can remove injured or senescent cells, and participate in the corresponding adaptive immune response [12, 13]. Toll-like receptor (TLR) belongs to the pattern recognition receptors (PRR). So far, human has a total of 11 TLRs family members (TLR1-11). Their genes locate in chromosome 4 (TLR1, 2, 3, 6, 10, 11), chromosome 9 (TLR4), chromo-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
TLR-4	CATCATACGGAGTAGGCCAAT	GACTATCCGGCTCATCGTC
TLR-9	GCCAGCCGAACTTGC	CGGAGTAGGCCAACTCA

some 1 (TLR5), chromosome 3 (TLR9), and chromosome X (TLR7, 8) [14]. Different TLRs can recognize and bind to different PAMPs (pathogen-associated molecular patterns). For instance, TLR4 mediates the recognition of lipopolysaccharides (LPS), while TLR9 mediates the recognition of bacterial DNA CpG sequences [15, 16]. However, TLR-4 and TLR-9 expressions in AAD and the regulatory role of Bifidobacterium have not been reported.

Materials and methods

Experimental animals

A total of 60 healthy SD rats at 2 months old and weighted 250 ± 20 g were bought from the Experimental Animal Center in Fujian Medical University and raised in SPF grade experimental animal center. The raising condition contained temperature at 21 ± 1°C, relative humidity at 50-70%, and 12 h day/night cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

Main reagents and instruments

Lincomycin hydrochloride and Bifidobacterium freeze-dried powder were purchased from Merck (USA). Pentobarbital sodium and lidocaine were bought from Zhpharma (Shanghai, China). Bifidobacterium, Lactobacillus, and Enterobacter specific medium was purchased from Invitrogen (USA). PVDF membrane was derived from Pall Life Sciences. Western blot related reagents were provided by Beyotime (Shanghai, China). ECL reagent was obtained from Amersham Biosciences. Rabbit anti rat NF-κB monoclonal antibody and goat anti rabbit HRP labeled IgG secondary antibody were provided by Cell Signaling (USA). RNA extraction kit and reverse transcription kit were purchased from Axygen (USA). Transwell chamber was obtained from Corning (USA). Labsystem Version 1.3.1

microplate reader was provided by BD (USA). ABI 7700 Fast real-time PCR amplifier was derived from ABI (USA). DNA amplifier was got from PE Gene Amp PCR System 2400.

Methods

Experimental animal grouping and diarrhea modeling: The rats were randomly equally divided into three groups with 20 in each group [17]. The rats in diarrhea group were treated by 4 ml lincomycin hydrochloride (0.3 g/ml) intragastric administration once a day for 7 days. The rats in Bifidobacterium were treated by 2 ml Bifidobacterium (1×10^{11} CFU/ml) intragastric administration at 4 h after lincomycin treatment once a day for 7 days. The rats in control were treated by equal amount of normal saline intragastric administration. The rats in diarrhea group presented spirits drooping, continuous diarrhea for 6-7 times/day, and weight loss after 3 days' lincomycin intragastric administration, suggesting successful modeling.

Sample collection: After treatment, the rat was killed and the mesenteric lymph nodes, intestine, and liver were extracted. The tissue was washed by PBS at 4°C for bacteria cultivation and -80°C storage, respectively.

Real-time PCR

Total RNA was extracted from the tissue by trizol and reversely transcribed to cDNA. The primers were designed using PrimerPremier 6.0 software and synthesized by Sangon (**Table 1**). Real-time PCR was performed at 52°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. GAPDH was selected as internal reference. The relative expression of mRNA performed by Real-time PCR was calculated by $2^{-\Delta\Delta Ct}$ method.

Western blot

The intestinal tissue was added with RIPA and cracked on ice for 15-30 min. Next, the tissues were treated by ultrasound at 5 s for 4 times and centrifuged at 10000 g for 15 min. The protein was transferred to new Ep tube and quantified by Bradford method. The protein was separated by 10% SDS-PAGE and transferred to PVDF membrane at 100 mA for 1.5 h. After
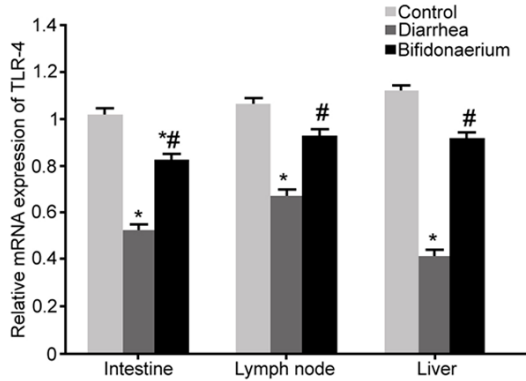
Figure 1. TLR-4 mRNA expression in different tissues. *$P < 0.05$, compared with control; #$P < 0.05$, compared with diarrhea group.
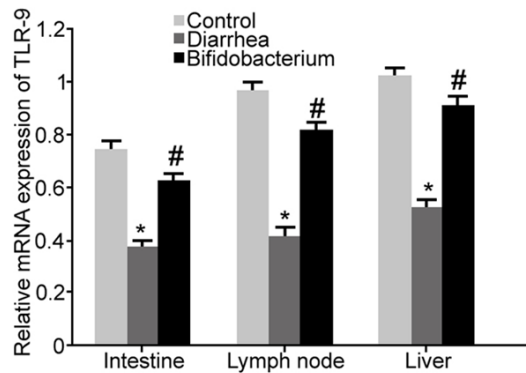

Figure 2. TLR-9 mRNA expression in different tissues. *$P < 0.05$, compared with control; #$P < 0.05$, compared with diarrhea group.

blocked by 5% skim milk for 2 h, the membrane was incubated in NF-κB monoclonal antibody (1:1000) at 4°C overnight. Then the membrane was incubated in goat anti rabbit secondary antibody (1:2000) at room temperature for 30 min. Next, the membrane was treated by developer for 1 min and exposed to observe the result. The film was scanned by Quantity One software and analyzed by protein image processing system. Each experiment was repeated for four times.

Bacteria cultivation

A total of 0.5 g mesenteric lymph nodes, intestine, and liver tissues were added with 4.5 ml diluent to prepare the concentration at 10^{-1}-10^{-8}. Next, the tissue at 10^{-6}, 10^{-7}, and 10^{-8} dilution was seeded in Bifidobacterium, Lactobacillus, and Enterobacter specific medium, respectively. The dilution was quantified by three drops. Colony formation in wet feces (CFU/g) was used to present the result.

Bacteria translocation criteria

According to the reference, bacterial translocation was observed based on liver and mesenteric lymph nodes [17]. The ratio of positive organ and total organ was treated as bacterial translocation rate.

Statistical analysis

All data analyses were performed on SPSS19.0 software. Measurement data were presented as mean ± standard deviation and compared by one-way ANOVA. P < 0.05 was depicted as statistical significance.

Results

TLR-4 mRNA expression in different tissues

Real-time PCR was applied to test TLR-4 mRNA expression in mesenteric lymph nodes, intestine, and liver. TLR-4 mRNA expression significantly reduced in mesenteric lymph nodes, intestinal, and liver from diarrhea rats compared with control (P < 0.05). Bifidobacterium treatment obviously reversed TLR-4 mRNA inhibition in mesenteric lymph nodes, intestine, and liver caused by AAD (P < 0.05) (**Figure 1**).

TLR-9 mRNA expression in different tissues

Real-time PCR was used to test TLR-9 mRNA expression in mesenteric lymph nodes, intestine, and liver. TLR-9 mRNA expression significantly reduced in mesenteric lymph nodes, intestinal, and liver from diarrhea rats compared with control (P < 0.05). Bifidobacterium treatment obviously reversed TLR-9 mRNA inhibition in mesenteric lymph nodes, intestine, and liver caused by AAD (P < 0.05) (**Figure 2**).

Intestinal flora changes analysis

Bacteria cultivation was selected to assess the impact of Bifidobacterium, Lactobacillus, and Enterobacter on common intestinal flora. Bifidobacterium, Lactobacillus, and Enterobacter amount markedly declined in diarrhea group compared with control (P < 0.05). Bifidobacterium treatment apparently increased Bifidobacterium, Lactobacillus, and Enterobacter amount compared with diarrhea group (P < 0.05) (**Figure 3**).
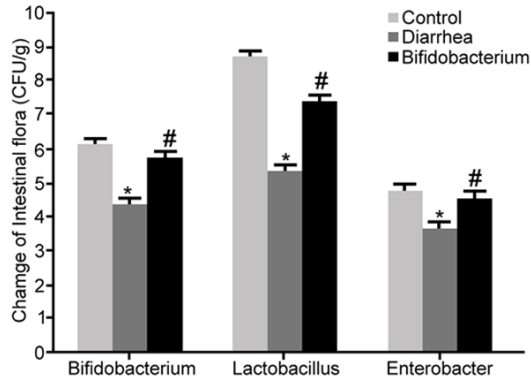
Figure 3. Intestinal flora changes analysis. *P < 0.05, compared with control; #P < 0.05, compared with diarrhea group.
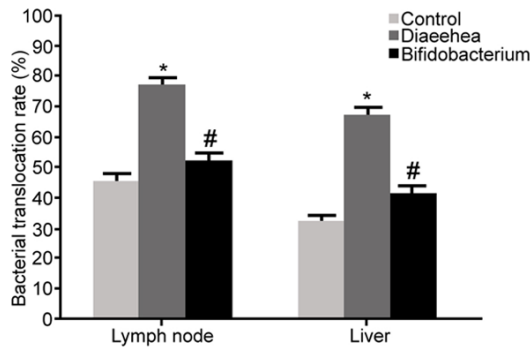

Figure 4. Bacteria translocation analysis. *P < 0.05, compared with control; #P < 0.05, compared with diarrhea group.

Bacteria translocation analysis

Bacteria translocation rate in mesenteric lymph nodes and liver was analyzed. The rate significantly elevated in diarrhea rats compared with control (P < 0.05). Bifidobacterium treatment obviously attenuated bacteria translocation rate compared with diarrhea group (P < 0.05) (**Figure 4**).

NF-κB expression in rat intestinal tissue

Western blot was used to analyze NF-κB protein expression in rat intestinal tissue. NF-κB protein markedly decreased in the intestinal tissue from diarrhea rats compared with control (P < 0.05). Bifidobacterium treatment apparently promoted NF-κB protein expression compared with diarrhea group (P < 0.05) (**Figure 5**).

Discussion

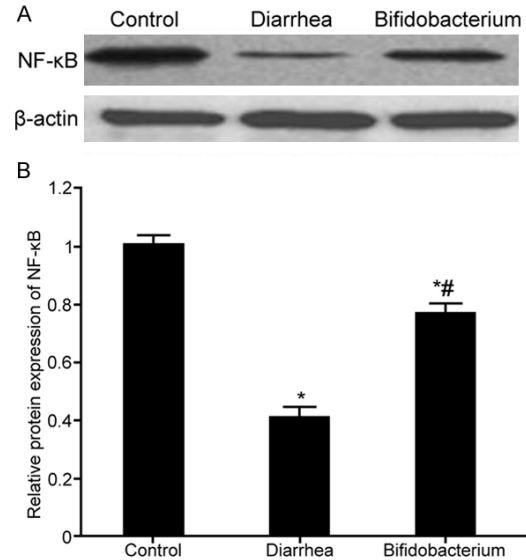Interdependence and mutual condition of intestinal normal flora sustains intestinal microecol-

Figure 5. NF-κB expression in rat intestinal tissue. A. Western blot detection of NF-κB expression; B. NF-κB protein expression analysis. *P < 0.05, compared with control; #P < 0.05, compared with diarrhea group.

ogy internal environment stability. AAD may induce intestinal flora disorder and bacterial translocation [18, 19]. Intestinal bacterial translocation mainly occurs in stress disorder or excess. Toxins or intestinal flora can pass through the intestinal mucosal barrier into the organ or tissue without flora, such as mesenteric lymph nodes and liver. Intestinal bacterial can directly translocate from the original site to the depths of the intestinal mucosa, and also to the surrounding, leading to tissue and organ injury [20, 21]. This study used lincomycin hydrochloride to establish rat AAD model, and confirmed that flora disorder and bacterial translocation occurs in AAD rat. It may be associated with the application of antibiotics, which leads to the microbes break through the damaged velum, resulting in bacterial translocation.

TLRs mainly express on the cell membrane. As an important member of PRR, TLRs play an important role in the immune system to identify pathogenic microorganisms [22]. TLR-4 participates in LPS recognition and signal transduction, while TLR-9 also plays an anti-inflammation role to protect intestinal epithelial cells damage and stabilize the intestinal environment [15, 16]. In this study, TLR-4 and TLR-9 expressions were suppressed in AAD, suggesting that immune function was damaged in diarrhea process, affecting immunity and bacterial

translocation in dynamic equilibrium [20, 23]. Bifidobacterium suppresses intestinal bacterial translocation by preventing adhesion and positioning of conditional pathogenic bacteria, helping intestinal mucosal barrier repair, and regulating the intestinal flora imbalance [24]. This study showed that Bifidobacterium promoted TLR-4 and TLR-9 expressions, improved innate immune, maintained intestinal flora balance, alleviated bacteria translocation, and enhanced NF-κB protein level in AAD rat. TLR-4 and TLR-9 deliver information to the cells and activate NF-κB to promote inflammatory cytokines IL-1 and IL-6 secretion, leading to the activation of innate immune [25]. However, the regulatory role and clinical application of Bifidobacterium in treating AAD remains further validation.

AAD inhibited TLR-4 and TLR-9 mRNA, induced intestinal flora disturbance and translocation and downregulated NF-κB expression. Bifidobacterium promoted TLR-4 and TLR-9 expression, improved innate immune, maintained intestinal flora balance, alleviated bacteria translocation and upregulated NF-κB expression. However, whether these effects of Bifidobacterium were through upregulation of NF-kB expression remains unclear and requires further studies.

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Disclosure of conflict of interest

None.

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