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Original Article

Effect of lactobacillus on Toll-like receptors expression and bacterial translocation in antibiotic diarrhea rats

Yangming Que[1,2], Mengli Gu[1], Feng Ji[1], Xinxin Zhou[1]

[1]Department of Gastroenterology, The First Affiliated Hospital of Medical College of Zhejiang University, Hangzhou, P. R. China; [2]Department of Gastroenterology, The Second Affiliated Hospital of Jiaxing University, Jiaxing, P. R. China

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Abstract: Diarrhea is a common digestive system disease. As a type of intestinal probiotics, lactobacillus plays a critical role in maintaining the normal function of intestinal tract. This study established a rat antibiotic diarrhea model to test Toll-like receptors expression, aiming to analyze the impact of lactobacillus on Toll-like receptors expression and bacterial translocation. A total of 60 Wistar rats were equally randomly divided into three groups. The rats in experimental group received lincomycin intragastric administration together with lactobacillus intervention. The rats in model group received lincomycin intragastric administration for 7 days to establish diarrhea model. General state and intestinal tissue changes under the microscope were observed. Bacterial translocation in intestine, liver, spleen, and mesenteric lymph nodes was tested by bacterial culture method. TLR2 and TLR4 mRNA and protein expression in small intestine cells were detected by RT-PCR and Western blot. The amplitude of weight loss in experimental group was lower than the model group. Bifidobacteria, lactobacillus, enterobacter, and enterococcus in intestine of the experimental group were obviously higher than those in the model group ($P < 0.05$). Bacterial translocation in liver, spleen, and mesenteric lymph nodes were significantly lower than those in the model group ($P < 0.05$). TLR2 and TLR4 mRNA and protein levels in experimental group was markedly higher than the model group and further elevated following time extension ($P < 0.05$). Lactobacillus intervention shows a protective effect on intestinal mucosal inflammation in diarrhea rat through increasing intestinal beneficial bacteria, inhibiting bacterial translocation, and enhancing TLR2 and TLR4 expression.

Keywords: Lactobacillus, diarrhea, Toll-like receptor, bacterial translocation

Introduction

Intestinal epithelial cells are important defensive line in the body that can timely resist the invasion of the intestinal bacteria and virus. There are numerous kinds of drugs for enteritis, but with the weakness of long treatment cycle, large side effect, and easy to relapse after stopping [1, 2]. Innate immunity is the first line of defense against pathogenic microorganism infection. It can effectively perceive the invasion of pathogenic microorganisms and induce immune response through specifically recognizing the conservative molecular structure of pathogens [3]. Toll-like receptor (TLR) expresses on the cell membrane. It is an important recognition receptor in the immune system that can identify a variety of pathogenic microorganisms [4, 5]. Up to now, there are about 13 kinds of TLR found by researchers. Only TLR2 and TLR4 can cause transcription factor translocation, activate immune related genes, release inflammatory factors, and thus induce inflammation. TLR level changes are important for the regulation of inflammatory reaction [6, 7]. TLR2, belonging to pattern recognition receptor (PRR), can recognize the cell wall of bacteria. TLR4 participates in a variety of signaling pathways mainly through recognizing lipopolysaccharide [8, 9]. It was found that TLR can identify the molecular composition of pathogenic microorganism and transfer the signal to the cells to activate NF-κB, leading to the release of inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, and TNF-α [10]. Lactobacillus is an important probiotics in the intestinal environment that can regulate intestinal flora, promote intestinal mucosa repair, and maintain the normal physiological function of intestinal tract to keep homeostasis. It was proposed that lactobacil-

lus can promote the intestinal normal flora engraftment and optimization by producing organic acids and reducing environmental pH value. Moreover, it also can produce protein similar to bacteria, thus having certain antibacterial function. On this basis, lactobacillus may generate hydrogen peroxide in the metabolic process, thus to activate hydrogen peroxide enzyme and kill the gram-negative bacteria [11]. This study established antibiotic diarrhea rat model by adopting lincomycin intragastric administration for 1 week. The experimental group received lactobacilli intervention for 2 weeks to observe the symptoms of diarrhea and weight loss, and intestinal mucosa pathological changes under the microscope. Bacterial translocation in the mesenteric lymph nodes, liver, and spleen were tested and compared. TLR2 and TLR4 mRNA and protein expression in small intestine cells were detected by RT-PCR and Western blot to analyze the influence of lactobacillus on TLR expression and bacterial translocation in diarrhea rat.

Materials and methods

Experimental animals

A total of 60 healthy male Wistar rats in SPF grade were enrolled, with mean age at 8 weeks and weighted 180±20 g. The rats were provided by the laboratory animal center of Zhejiang University.

Rats were used for all experimentsand all procedures, which were approved by the Animal Ethics Committee of the First Affiliated Hospital of Medical College of Zhejiang University.

Experimental drugs

Lactobacillus freeze-drying powder was from XinYi pharmaceutical factory (Shanghai). TLR2 and TLR4 blocking buffer and rabbit anti mouse secondary antibody were from Keygentec. TLR2 and TLR4 monoclonal antibodies were from Biogot. TRIzol reagent was from Invitrogen. PCR kit was from Takara.

Methods

Rat antibiotic diarrhea model establishment: A total of 60 Wistar rats were equally randomly divided into three groups as follows: The rats in the experimental group received 4 ml lincomycin daily via intragastric administration for 7 days, together with 4 ml lactobacillus intragastric administration every 4 h for 14 days. The rats in the model group received 4 ml lincomycin daily intragastric administration for 7 days. The rats in the blank group received equal amount of normal saline daily via intragastric administration for 7 days.

Specimen collection: The rats were killed after successfully modeling. A total of small intestine tissue at 5-6 cm from ileocecal junction, liver, spleen, and mesenteric lymph nodes were collected and stored at -80°C.

Bacterial culture: About 0.5 g of intestinal content, liver, spleen, and mesenteric lymph nodes were collected and diluted for ten times. Bifidobacterium, lactobacillus, enterobacter, and enterococcus were cultured by Mile and Misra instillation method. The bacteria were anaerobic cultured at 37°C in Bs and Ls medium for 48 h, or aerobic cultured at 37°C in EMB and EC medium for 48 h for calculation. Bacterial isolated from liver, spleen and lymph node were resuspended in PBS followed by seeded into culture plate or gram-negative plate and subsequent culture for 24 hours at 37°C. Then the colony number of bacterial was calculated. Translocation rate was calculated as a ratio of the colony number of bacterial growth on culture plate to bacterial growth on gram-negative plate.

Western blot: A total of 40 μg protein isolated from intestine tissue was separated by 8% SDS-PAGE and transferred to membrane. After blocked for 1 h, the membrane was incubated in diluted primary antibody for 30 min (TLR2 and TLR4, 1:200; β-actin, 1:500) and then incubated in secondary antibody for 1 h (1:2000). At last, the membrane was developed and analyzed. The protein level was quantified in relative to the control (β-actin) which was represented as a ratio of β-actin to target protein.

RT-PCR: Total RNA was extracted from intestinal tissue and reverse-transcripted to cDNA. The primers used were listed in **Table 1**. The reaction condition was composed of 95°C for 30 s, 95°C for 5 s, and 40 cycles of 60°C for 34 s, 95°C for 15 s, and 60°C for 1 min, and at last 95°C for 15 s. Target gene mRNA relative expression was calculated as a ratio normalized against internal control GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers sequence

Gene	Sequence	Product length
TLR2		370 bp
Forward	5'-AAA CGG TAA CAA TAC GGA G-3'	
Reverse	5'-TGA CAA CTG TCG GGC ATA-3'	
TLR4		410 bp
Forward	5'-CAG AGC CGT TGG TGT ATC-3'	
Reverse	5'-CCC TGT GAG GTC GTT GA-3'	
β-actin		150 bp
Forward	5'-AGT TGC GTT ACA CCC TTT C-3'	
Reverse	5'-CAC CTT CAC CGT TCC AGT-3'	

Table 2. Weight changes of rats in different groups

Group	Before modeling	After modeling	Increasing amplitude
Experimental group	8.63±0.75	8.02±0.22*,#	-0.61±0.53*,#
Model group	8.64±0.87	6.53±0.75#	-2.16±0.28#
Blank group	8.04±0.73	10.22±0.86	2.18±0.13

Data were shown as mean±SD. *P < 0.05, compared with model group. #P < 0.05, compared with blank group.

Statistical analysis: Data analysis was performed on SPSS17.0 software. Enumeration data was compared by chi-square test, while measurement data was compared by ANOVA with Newman-Keuls multiple comparison post-hoc analysis. The data was depicted as mean±standard deviation. P < 0.05 was adopted as significance level.

Results

General state

The rats in the model group appeared abdominal distention, yellow watery stools, poor eating, less activity, sluggish and obvious weight loss after modeling. The symptom was most severe on the fifth day. The rats in experimental group also exhibited abdominal distension and diarrhea, while the amplitude of weight loss was lower than model group. The rats in blank group presented normal eating and defecate (**Table 2**).

Intestinal tissue changes under microscope

Intestinal mucosa appeared hyperemia, edema, erosion, bleeding in model group, together with a large amount of neutrophils, lymphocytes, and eosinophils infiltration influencing the submucosa. The experimental group exhibited slighter intestinal mucosal hyperemia and edema. The intestinal mucosa in the blank group was normal (**Figure 1**).

Intestinal flora comparison

Bifidobacteria, lactobacillus, enterobacter, and enterococcus in intestine of experimental group were lower than the blank group but obviously higher than the model group (P < 0.05). Bifidobacteria and lactobacillus contents reduced on the seventh day but lack of significant difference compared with blank group (P < 0.05) (**Table 3**).

Bacterial translocation comparison

The bacterial translocation rate of liver, spleen, and mesenteric lymph nodes in experimental group was significantly lower than the model group on the 3rd and 7th day (P < 0.05). No bacterial translocation in liver, spleen, and mesenteric lymph nodes in experimental group was observed on the 7th day (P < 0.05) (**Table 4**).

TLR2 and TLR4 protein expression in small intestine

TLR2 and TLR4 protein expression in experimental group were obviously higher than the model group (P < 0.05), while their levels in experimental group on the 7th day were significantly higher than on the 3rd day (P < 0.05) (**Figure 2**; **Table 5**).

TLR2 and TLR4 mRNA expression in small intestine

TLR2 and TLR4 mRNA expression in experimental group were markedly higher than the model group (P < 0.05), while their levels in experimental group on the 7th day were apparently higher than on the 3rd day (P < 0.05) (**Table 6**).

Discussion

Intestinal microenvironment changes can cause intestinal normal flora disorder. Pathogenic

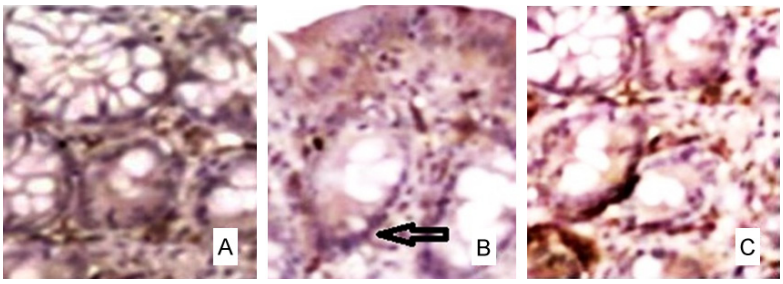

Figure 1. Intestinal tissue expression under microscope (×200). A: experimental group. B: model group. C: blank group. Arrow shows bleeding or hyperemia.

Table 3. Intestinal flora comparison

Group	Experimental group	Model group	Blank group
Bifidobacteria			
3rd day	6.67±0.25*	5.37±0.24#	8.28±0.38
7th day	8.50±0.24&	5.04±0.37#	8.59±0.25
Lactobacillus			
3rd day	7.18±0.28*	6.24±0.24#	8.06±0.27
7th day	8.35±0.25&	6.51±0.25#	8.24±0.21
Enterobacter			
3rd day	6.06±0.12*	5.53±0.16#	6.17±0.26
7th day	6.36±0.23&	6.42±0.25#	6.29±0.19
Enterococcus			
3rd day	6.15±0.26*	5.23±0.26#	6.37±0.24
7th day	6.60±0.27&	6.45±0.38#	6.58±0.30

Data were shown as mean±SD. *P < 0.05, compared with model group. #P < 0.05, compared with blank group. &P < 0.05, compared with 3rd day.

microorganisms' invasion at this time may lead to bacterial translocation [12]. Intestinal probiotics commonly used in clinic, including lactobacillus and bifidobacterium, can maintain intestinal normal function, repair the defense capability of the barrier, and regulate intestinal normal flora [13]. This study established antibiotic diarrhoea animal model and adopted lactobacillus intervention to analyze its impact on TLR expression and bacterial translocation.

In this study, lincomycin intragastric administration was applied to establish diarrhea animal model. Lactobacillus intervene was adopted in the experimental group. The rats in the model group appeared abdominal distention, yellow watery stools, poor eating, less activity, sluggish, and obvious weight loss after modeling. The rats in experimental group also exhibited abdominal distension and diarrhea, while the amplitude of weight loss was lower than model group. Intestinal mucosa appeared hyperemia, edema, erosion, and bleeding in model group, together with a large amount of neutrophils, lymphocytes, and eosinophils infiltration influencing the submucosa. The experimental group exhibited slighter intestinal mucosal hyperemia and edema, conforming to the clinical symptoms and microscopic performance.

Intestinal probiotics can maintain the balance of intestinal flora. Its reduction may lead to intestinal flora disorder, bacterial translocation, and endotoxin elevation, and intestinal mucosal barrier damage [14]. Bifidobacterium and lactobacillus are the representatives of dominant bacterial group in normal intestinal tract [15]. Bifidobacteria, lactobacillus, enterobacter, and enterococcus in intestine of experimental group were lower than the blank group but obviously higher than the model group on the 3rd day. Bifidobacteria and lactobacillus contents were lack of significant difference compared with blank group on the 7th day. It suggested that bifidobacteria and lactobacillus reduced most significantly in antibiotic diarrhea induced intestinal flora disorder. The phenomenon of dominant bacterial group reduction was more seriously following time extension, while lactobacillus can improve the structure of normal intestinal flora in diarrhea rat and maintain the homeostasis.

Intestinal mucous barrier function weakened during stress state, while some intestinal endotoxin or exogenous microbes may invade tissues out of intestine, such as lymph nodes, liver, spleen, which is called bacterial translocation. Bacterial translocation is divided into horizontal and vertical types. Among them, the horizontal translocation refers to the flora shift to periphery from in situ, while the vertical translocation means the flora shift to intestinal mucosa from in situ [16, 17]. This study examined bacterial translocation in different organs. The bacterial translocation rate of liver, spleen, and mesenteric lymph nodes in experimental group

Table 4. Bacterial translocation comparison

Group	Experimental group	Model group	Blank group
Liver			
3rd day			
Bacterial count (CFU/g)	2.12±0.11*,#	3.45±0.14#	0
Translocation rate (%)	17*,#	50#	0
7th day			
Bacterial count (CFU/g)	0*	2.21±0.12#,&	0
Translocation rate (%)	0*,&	33#,&	0
Spleen			
3rd day			
Bacterial count (CFU/g)	0*	3.05±0.02#	0
Translocation rate (%)	0*	33#	0
7th day			
Bacterial count (CFU/g)	0	0#,&	0
Translocation rate (%)	0	0#,&	0
Mesenteric lymph nodes			
3rd day			
Bacterial count (CFU/g)	2.52±0.33*,#	3.46±0.21#	0
Translocation rate (%)	67*,#	83#	0
7th day			
Bacterial count (CFU/g)	0*,#,&	2.41±0.11#,&	0
Translocation rate (%)	0*,#,&	33#,&	0

*P < 0.05, compared with model group. #P < 0.05, compared with blank group. &P < 0.05, compared with 3rd day.

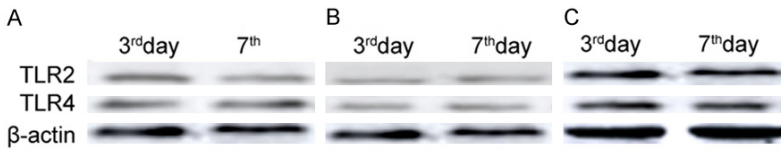

Figure 2. TLR2 and TLR4 protein expression in small intestine. A: experimental group. B: model group. C: blank group.

was significantly lower than the model group on the 3rd and 7th day. No bacterial translocation in liver, spleen, and mesenteric lymph nodes in experimental group was observed on the 7th day, indicating lactobacillus intervention can alleviate intestinal bacterial translocation in antibiotic diarrhea rat.

TLRs are important transmembrane recognition receptors that expressed in intestinal epithelial cells. They can accurately identify the biomarker on the membrane of pathogen, trigger inflammation, and regulate the phagocytosis of phagocytes [18-20]. This experiment measured TLR2 and TLR4 protein expression in small intestine. TLR2 and TLR4 protein expression in experimental group were obviously higher than the model group, while their levels in experimental group on the 7th day were significantly higher than on the 3rd day. Further analysis showed that TLR2 and TLR4 mRNA expression in experimental group were markedly higher than the model group, while their levels in experimental group on the 7th day were apparently higher than on the 3rd day. Previous studies pointed out that bifidobacteria intervention declined TLR2 and TLR4 expression in the terminal ileum of rats with ileitis with dose dependence [21-23]. TLR can quickly identify the corresponding ligands in a short time once the intestinal mucosa was attacked by toxins. They may trigger immune response through complicated signaling pathway, eventually inducing intestinal mucosal inflammatory reaction and exogenous lactic acid bacteria engraftment in the intestine, inhibiting potential pathogenic bacteria excessive proliferation, reducing bacterial translocation, and controlling endotoxin generation and release. They also can synthesize glutamine to promote damaged intestine recovery. Lactobacillus may have a certain protective effect on intestinal mucosal inflammation caused by TLR2 and TLR4.

Lactobacillus intervention may increase the content of bifidobacteria, lactobacillus, enterobacter, and enterococcus, inhibit bacterial translocation in liver, spleen, and mesenteric lymph nodes, and upregulate TLR2 and TLR4 expression in antibiotic diarrhea rat. Lactobacillus may play a protective role in TLR induced intestinal mucosa inflammation. Lactobacillus may regulate intestinal microenvironment in diarrhea rat to play a treatment role.

Table 5. TLR2 and TLR4 protein expression in small intestine

Group	TLR2		TLR4	
	3rd day	7th day	3rd day	7th day
Experimental group	0.56±0.05*	0.64±0.047*,&	0.044±0.07*	0.058±0.04*,&
Model group	0.054±0.04#	0.048±0.04#,&	0.042±0.05#	0.037±0.04#,&
Blank group	0.067±0.08	0.073±0.1	0.61±0.05	0.72±0.06

Data were shown as mean±SD. *P < 0.05, compared with model group. #P < 0.05, compared with blank group. &P < 0.05, compared with 3rd day.

Table 6. TLR2 and TLR4 mRNA expression in small intestine

Group	TLR2 mRNA		TLR4 mRNA	
	d3	d7	d3	d7
Experimental group	0.74±0.05*	0.98±0.068*,&	0.81±0.02*	1.06±0.04*,&
Model group	0.52±0.02#	0.25±0.02#,&	0.62±0.03#	0.31±0.01#,&
Blank group	1.19±0.04	1.35±0.06	0.94±0.05	1.25±0.07

Data were shown as mean±SD. *P < 0.05, compared with model group. #P < 0.05, compared with blank group. &P < 0.05, compared with 3rd day.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Feng Ji, Department of Gastroenterology, The First Affiliated Hospital of Medical College of Zhejiang University, 79 Qingchun Road, Hangzhou 310006, Zhejiang, P. R. China. Tel: +86-571-87236114; Fax: +86-571-87236114; E-mail: fengjizxc@163.com

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