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Protective effects of bifidobacteria on intestines in newborn rats with necrotizing enterocolitis and its regulation on TLR2 and TLR4

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ABSTRACT. We established a necrotizing enterocolitis (NEC) rat model and explored the role of bifidobacteria in the intestines of the rats and its regulation on intestinal Toll-like receptors (TLRs). Seventy-five newborn Sprague-Dawley rats were randomly divided into 5 groups (15 rats/group): group A, artificial feeding group (formula-fed); group B, NEC model (LPS + formula-fed); group C, bifidobacterium (LPS + formula-fed + bifidobacterium microcapsules, intragastric administration); group D, artificial feeding + bifidobacterium (formula-fed + bifidobacterium microcapsules gavage); group E, rat breast-feeding group (rat breast-feeding). After 3 days of feeding, rats were placed in incubators, fasted for 12 h, and killed by decapitation. The ileocecal proximal segment ileum was fixed and sliced; pathological examination was conducted, and TLR2, TLR4, and nuclear factor-κB p65 protein expression in the intestinal tissue was detected by immunohistochemistry. There was a statistically significant difference

in pathological scores between groups C and B (H = 21.789, P = 0.000), and the former was lower than the latter. TLR2, TLR4, and nuclear factor-κB p65 expression in intestinal tissue was determined in groups A-E. There were statistically significant differences between groups C and B (P = 0.001; P = 0.000; P = 0.000). Bifidobacteria had a protective effect on the intestines of newborn rats with NEC, which showed reduced NEC and intestinal damage severity. This observation may be related to the reduced levels of TLR2, TLR4, and nuclear factor-κB P65 observed during the inflammatory response.

Key words: Bifidobacteria; Enterocolitis; Necrotizing; Newborn; Rats; Toll-like receptor

INTRODUCTION

Neonatal necrotizing enterocolitis (NEC) is an inflammatory bowel and necrotizing emergency that occurs during the neonatal period, with the main pathological manifestation of inflammatory hemorrhagic necrosis in the terminal ileum and proximal colon. Although numerous studies have examined NEC, its etiology and pathogenesis remain unclear, and effective control methods are lacking. Previous studies showed that in addition to preterm birth, NEC is also related to other potential predisposing factors such as intestinal hypoxic-ischemic injury, formula feeding, and pathologic bacterial colonization (An et al., 2007). In the presence of various risk factors, changes in gut microflora and intestinal epithelial permeability are increased, the intestinal barrier is damaged, and bacterial products such as lipopolysaccharide (LPS) invade and activate the immune system to cause an inflammation cascade reaction, resulting in intestinal damage.

Bifidobacterium is the dominant bacterial genus in normal neonatal intestinal flora (commensal bacteria) and has received attention for preventing NEC and inflammatory bowel disease. In the 2011 Yale Summit of Probiotic Use, probiotic was firstly recommended for NEC; the strains were *Lactobacillus* NCDO174813 and *Bifidobacterium* NCDO14534788 (Floch et al., 2011). However, some physicians have stated that because of the unclear specific molecular mechanism of the protective effect of probiotics in the intestine and the risk of intestinal infection, probiotics should be used cautiously. Therefore, it is important to clarify further the role of probiotics in intestinal molecular mechanisms in order to regulate the use of probiotics.

In this study, we established a joint artificial feeding LPS-induced neonatal NEC rat model and conducted bifidobacterium intervention to observe the effects of bifidobacteria on neonatal rats with NEC and the expression of intestinal Toll-like receptor-2 (TLR2), TLR4, nuclear factor (NF)-κ gene-binding p65 (NF-κB p65 nuclear) to provide an experimental basis for clinical application of bifidobacteria in NEC prevention.

MATERIAL AND METHODS

Reagents

LPS (*Escherichia coli* O55: B5) was purchased from Sigma (St. Louis, MO, USA). *Bifidobacterium* microcapsules were purchased from Guangzhou Institute of Microbiology

(Guangzhou, China). The NF-κB p65 immunohistochemistry primary antibody (rabbit anti-rat) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The TLR2 immunohistochemistry primary antibody (rabbit anti-rat) was purchased from GenWay Biotech, Inc. (San Diego, CA, USA). TLR4 immunohistochemistry primary antibody (rabbit anti-rat) was purchased from Abcam (Cambridge, UK). The universal IgG antibody-horseradish peroxidase multimers (goat anti-rabbit) and DAB color kit were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Experimental animals and groups

Seventy-five newborn specific pathogen-free Sprague-Dawley rats within 2 h after birth were provided by the Guangdong Medical Laboratory Animal Center [animal license No.: SCXK (Yue) 2008-0002]. Neonatal rats, weighing 4-7 g, were male or female and were numbered in the descending order of their weight. Rats were randomly divided into 5 groups (15 rats/group) by balloting: group A was the artificial feeding group (formula-fed); group B was the NEC model group (LPS + formula-fed); group C was the bifidobacterium group (LPS + formula-fed + bifidobacterium microcapsules, intragastric administration); group D was the artificial feeding + bifidobacterium group (formula-fed + bifidobacterium microcapsules gavage); and group E was the rat-breast feeding group (rat breast-feeding). The LPS dose was 30 mg/kg; neonatal rats in groups A, B, C, and D were separated from the mother at birth and placed in an incubator for artificial feeding; rats in group E were breast-fed by the mother.

Modeling

Preparation of milk substitutes

Based on a previous study (Newman and Hammond, 2010), the detailed milk substitute recipe was 4.60 g Nestle Liduojing 1 segment milk, 8.68 g protein powder, 49.2 mL fat emulsion (C14-24), and sterile distilled water to 100 mL. The calorie count of this mixture amounted to 581 kJ calories (Table 1).

Table 1. Milk replacer of the rat and mouse milk composition.

Component	Milk replacer	Neonatal rat
Sugar (g/L)	26.5	23-27
Protein (g/L)	74.2	73-77
Fat (g/L)	110	108-112
Quantity of heat (kJ/L)	5810	5780-5920

Artificial feeding methods

Neonatal rats in groups A, B, C, and D were separated from the mother at birth and placed in an incubator with a light and dark cycle of 12/12 h; breeding and experiments were conducted in Experimental Animal Center of Guangzhou Medical University (in accordance with the specific pathogen-free level requirements) and artificial milk was used for feeding.

Feeding practices referred to the literature (Mshvildadze and Neu, 2009) with some modifications. Before intragastric administration, a 24-G intravenous catheter sleeve was disinfected with 75% alcohol and washed with saline followed by medical paraffin oil for lubrication, to facilitate insertion into stomach, and then a 1-mL syringe was connected. Newborn rats were held with the left hand, and a medical cotton swab was dipped in saline and 75% alcohol to clean and disinfect the skin around the mouth. After cleaning, keeping the mouse's head slightly back and the mouth open, a 1-mL syringe was connected to the casing and a hose was inserted into the stomach slowly through mouth and esophagus to a depth of approximately 2.5-3 cm. At 2 h after birth, milk was given 0.1 mL/time per 4 h, increasing by 0.05 mL every 12 h and with continuous feeding for 72 h, increasing by no more than 0.3 mL each time. When there was residual milk, milk = need milk - remnant milk in stomach. After successful intragastric administration, stomach foam filling could be seen through the abdominal wall, which contained white emulsion. After intragastric administration, newborn rats were placed back into the incubator. The gastric tube was sterilized. Group B was fed with conventional milk formula; group C was given bifidobacterium-fortified formula milk: 1 g triturated bifidobacterium microcapsules (1 x 10^{11} CFU) (Guangzhou Institute of Microbiology) was added to 10 mL milk substitutes (1 x 10^{10} colony-forming units/mL) once daily. Feeding began 2 h after birth, and milk was given 0.1 mL/time per 4 h, increasing by 0.05 mL every 12 h, which was followed by continuous feeding for 72 h, increasing by no more than 0.3 mL each time. Rats were fasted for 12 h after the last feeding and then killed by decapitation; the terminal ileum tissue was collected.

LPS intragastric administration

LPS was diluted to 2 mg/mL with sterile water; intragastric administration was conducted once daily at a dose of 30 mg/kg and continued for 3 days. The formula feeding amount was calculated as required milk - LPS - gastric residual milk.

Intestinal pathological examination

The terminal ileum tissue was fixed in 10% formalin; after paraffin-embedding and slicing, morphological changes were observed under light microscope following hematoxylin and eosin staining. Based on the pathological scoring criteria set by Ganguli and Walker (2011) (Table 2), terminal ileum tissue injury was scored by 2 professional pathologists under double-blind conditions. The highest score was used to determine the extent of damage; histological score ≥2 points was recognized as NEC.

Table 2. Newborn mice intestinal damage criteria.

Number of points	Pathological changes
0	Intestinal mucosa villus complete, the structure is normal
1	Minor submucosal and/or lamina propria swelling separation
2	Moderate submucosal and/or inherent layer separation, submucosal edema and/or muscular layer
3	Severe submucosal and/or inherent layer separation, submucosal edema and/or muscular layer, the local fluff off
4	Intestinal villus disappeared with intestinal necrosis

Detection of TLR2, TLR4, and NF-κB p65 expression in intestinal tissue by immunohistochemistry

The ileocecal proximal ileum was fixed and sliced and immunohistochemistry was used to detect the expression levels of TLR2, TLR4, and NF-κB p65 in intestinal tissue. The distribution of TLR2, TLR4, and NF-κB p65 was observed under a light microscope. Five different horizons were randomly selected for each slice; using the image Pro Plus image analysis software, brown areas in the cytoplasm or nucleus tissue were selected in HSI format: H: 0-11, S: 0-255, I: 0-255. After macro compilation, semi-quantitative analysis of the immunoreactivity tissues of TLR2, TLR4, and NF-κB p65 was performed and the mean integrated optical density/area (density mean) was calculated. The mean and standard deviation of slices were calculated for each group. To ensure the reliability of the experimental results, all slices were observed under a microscope under the same conditions.

Statistical analysis

SPSS 13.0 statistical package was used for data analysis (SPSS, Inc., Chicago, IL, USA); the results are reported as means ± standard deviation. The Kruskal-Wallis H-test analysis was used for ileum pathological scoring. TLR2, TLR4, and NF-κB p65 expression differences were analyzed using analysis of variance; $P < 0.05$ was considered to be statistically significant.

RESULTS

Pathological changes in intestinal tissue of neonatal rats in each group

Light microscopy images of the histopathological features of the intestines in the neonatal rats in groups A, B, C, D, and E are shown in Figure 1. The incidences of NEC in each group were as follows: group A, 20% (3/15); group B, 73% (11/15); group C, 27% (4/15); group D, 13% (2/15); and group E, 0% (0/15). Pathology scores in each group are shown in Table 3. Pathology scores for groups B and C were compared with group E, which revealed statistically significant differences ($H_{B-E} = 27.406$, $P_{B-E} = 0.000$; $H_{C-E} = 5.513$, $P_{C-E} = 0.009$); there was also a statistically significant difference between groups C and B in pathology scores ($H = 8.119$, $P = 0.003$).

Immunohistochemical detection of TLR2, TLR4, and NF-κB p65 expression in neonatal intestinal tissues of rats

Compared with group E, the expression level of TLR2, TLR4, and NF-κB p65 in intestinal tissues of group B showed statistically significant differences ($P = 0.011$; $P = 0.000$; $P = 0.000$). Compared with group A, the expression level of TLR2, TLR4, and NF-κB p65 in intestinal tissues of group B showed statistically significant differences ($P = 0.021$; $P = 0.000$; $P = 0.000$). Compared with group E, the expression level of TLR2, TLR4, and NF-κB p65 in intestinal tissues of group C showed statistically significant differences ($P = 0.056$; $P = 0.493$; $P = 0.066$). Compared with group B, the expression level of TLR2, TLR4, and NF-κB p65 in

intestinal tissues of group C showed statistically significant differences (P = 0.001; P = 0.000; P = 0.000). The optical density value of each group is shown in Table 4. The expression characteristics of TLR2 in neonatal rat intestinal tissue in groups A, B, C, D, and E are shown in Figure 2. The expression characteristics of TLR4 in neonatal rat intestinal tissue in groups A, B, C, D, and E are shown in Figure 3. The expression characteristics of NF-κB p65 in neonatal rat intestinal tissue in group A, B, C, D, and E group are shown in Figure 4.

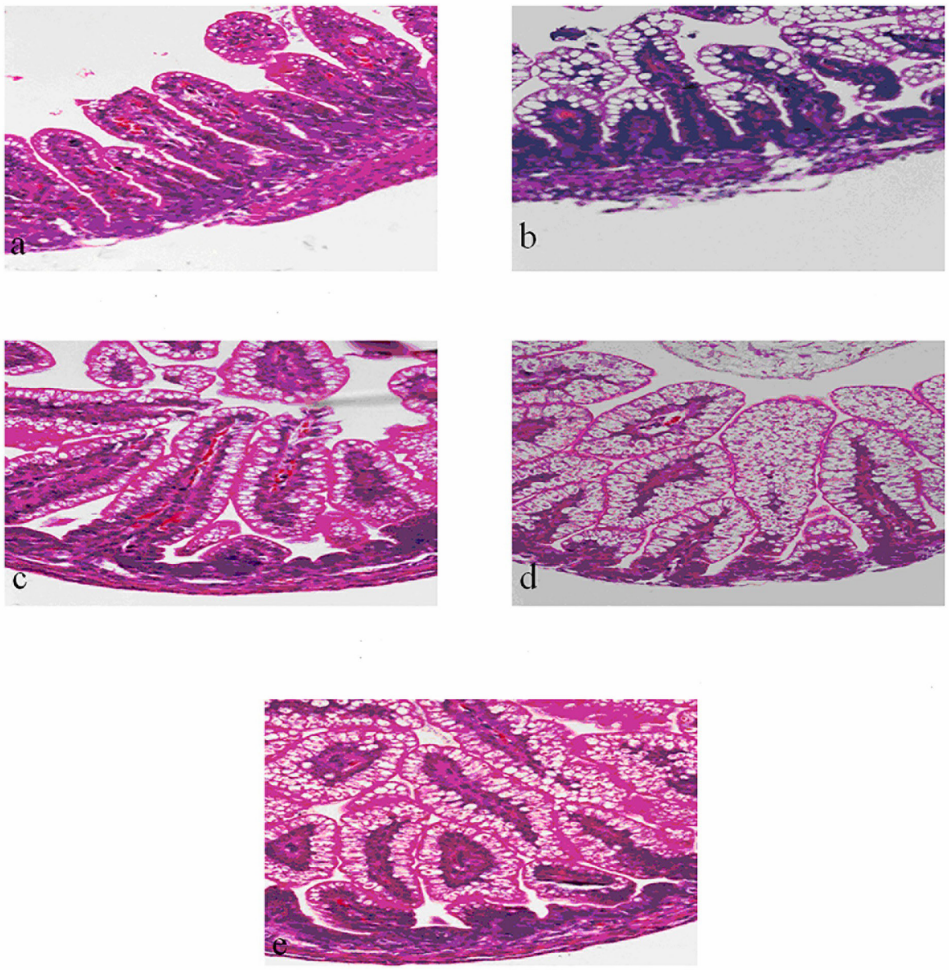

Figure 1. Intestinal pathological features of neonatal rats in each group under an optical microscope (H&E 200X). **a.** In Group A, there were slight separation and swelling of submucosal and/or the intrinsic layer and fluff edema, and epithelial shedding is limited to the top of the pile; **b.** In Group B, fluff mixed, partial hair loss and hair missing, submucosal and/or muscular edema, and lamina propria and muscular layers were separated; **c.** In group C, there were epithelial edema and pithelial shedding in the top of part villus; **d.** In group D, there was a slight swelling and separation of submucosal and/or the intrinsic layer, no epithelium; **e.** In E group, the structural integrity of intestinal villi and muscular integrity were observed; intestinal glands arranged regularly; no edema was observed in the mucous layer, submucosa, and lamina propria.

Table 3. Pathological grading comparison between groups (means ± SD).

Group	N	Intestinal tissue injury score
A	15	1.75 ± 0.37
B	15	3.37 ± 0.27
C	15	1.53 ± 0.44
D	15	0.92 ± 0.39
E	15	0.30 ± 0.18
H value		70.290
P value		0.000

Table 4. Immunohistochemical detection of each group with the newborn rat intestinal tissue TLR2 and TLR4, NF-KB seem p65 average optical density (means ± SD).

Group	N	TLR2	TLR4	NF-κB p65
A	15	0.33 ± 0.05	0.36 ± 0.03	0.35 ± 0.02
B	15	0.35 ± 0.05	0.48 ± 0.05	0.43 ± 0.03
C	15	0.30 ± 0.03	0.34 ± 0.03	0.29 ± 0.03
D	15	0.30 ± 0.02	0.37 ± 0.04	0.32 ± 0.02
E	15	0.29 ± 0.03	0.35 ± 0.02	0.30 ± 0.02
H value		8.803	38.599	75.076
P value		0.000	0.000	0.000

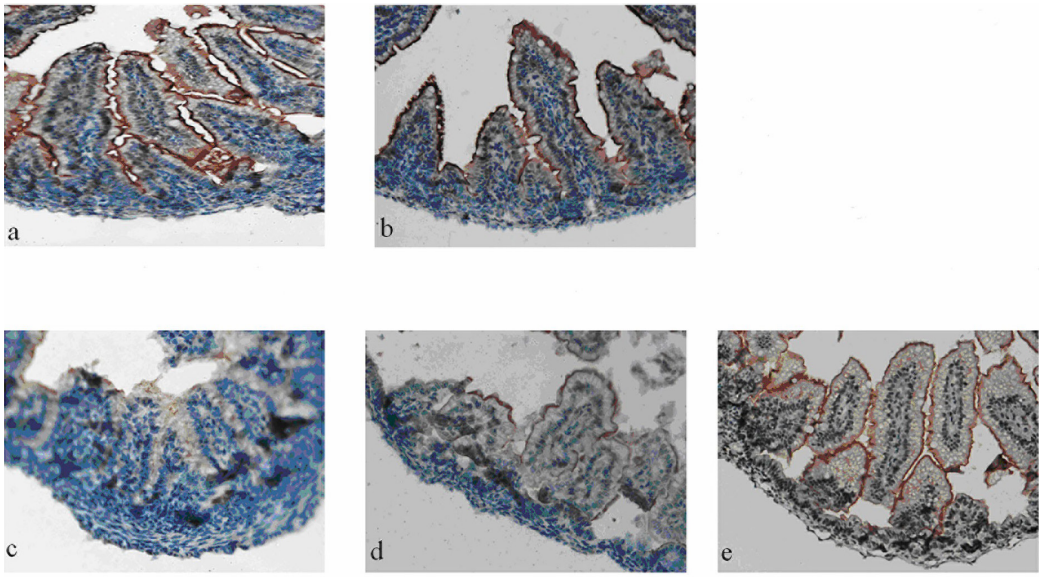

Figure 2. Immunohistochemistry can detect the expression level of TLR2 in neonatal intestinal tissues of rats in each group (200X). TLR2 was expressed in each group, and was more obvious in crypts. There was weakly positive staining on the top of intestinal epithelial cytoplasm in groups C (**c**), D (**d**), and E (**e**), while there was strong positive expression on villi and crypts in groups B (**b**) and A (**a**).

DISCUSSION

When a baby is born, symbiotic bacteria gradually colonize the sterile gut. During the process of bacterial colonization into the immature gut, intestinal flora disorder and innate immune dysregulation can increase the incidence of NEC. TLRs are cell transmembrane recep-

tors of the natural immune system, and they play an important role in preventing the entry of extracellular pathogens. TLR2 can not only identify Gram-positive bacterium peptidoglycan and lipoteichoic acid, but can also can combine with CD14, which improves the identification and transduction of the Gram-negative bacterium cell wall LPS (Kirschning et al., 1998). Gram-negative bacterium LPS are ligands of TLR4. Different TLRs, by recognizing their respective pathogens, trigger signal pathways, thereby activating the immune system, resulting in either immune tolerance or disease induction.

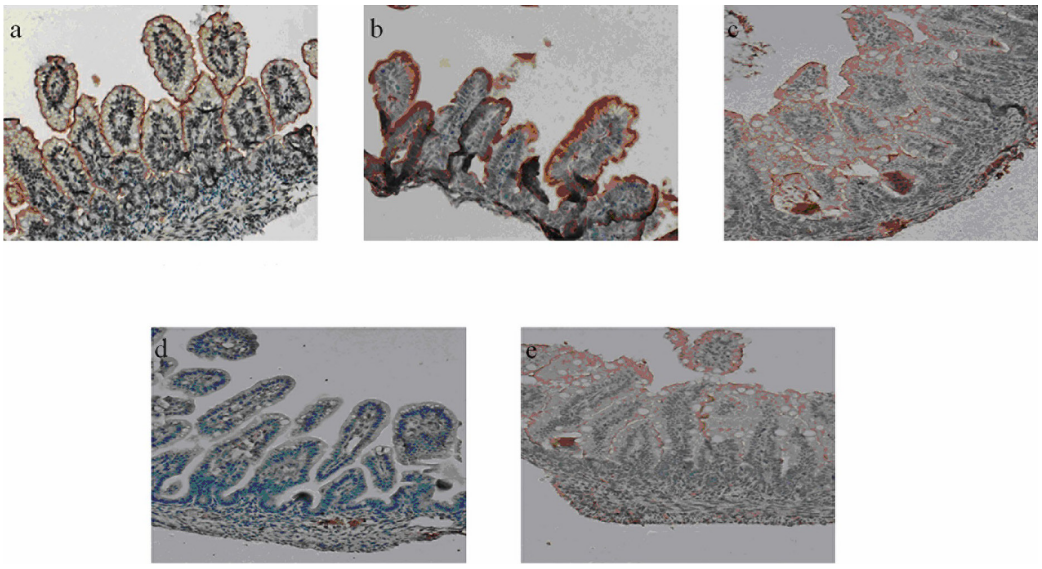

Figure 3. Immunohistochemistry can detect the expression level of TLR4 in neonatal intestinal tissues of rats in each group (200X). TLR4 in groups C (**c**), D (**d**) and E (**e**) was only weak expressed in rat intestinal epithelial cells, and not expressed in the submucosa. There was an enhanced and increased expression in group A (**a**), while in group B (**b**) the expression was significantly increased, showing diffuse or granular distribution.

NF-κB is a transcription factor in eukaryotes present in nearly all nucleated cells. NF-κB typically refers to the p65/p50 dimer. The carboxyl terminus of p65 contains a binding site involved in promoting transcription activity. Under normal circumstances, intestinal epithelial cells only express a very small amount of TLR2 and TLR4, and thereby monitor the state of the normal intestinal flora, while NF-κB can also remain in an inactive state in the cytoplasm. When intestinal epithelial cells are stimulated by bacteria, the bacterial component or its product or cytokine acts through TLRs and a series of signal transduction pathways to transfer NF-κB into the nucleus. NF-κB recognizes and binds to a number of genes in the inflammation specific promoter sequence. Through transcription and translation, cytokines involved in the immune response and inflammatory reactions are over-expressed. In an animal model for stimulated-LPS, a previous study showed that the activated TLR4 pathway, by causing an inflammation cascade reaction, regulated the balance of intestinal injury repairing in neonatal animals and promoted the development and progression of NEC (Afrazi et al., 2011). Other studies indicated that when NEC occurs, TLR4 expression was increased, while TLR4 expression was decreased (e.g., to polyunsaturated fatty acids), which can help reduce the incidence

or severity of NEC (Lu et al., 2007). Liu et al. (2009) showed that when newborn rats were given artificial feeding and subjected to hypoxic conditions, the content of ileum TLR2 was significantly increased. Growth occurred before the pathological changes in intestinal injury, which may explain TLR2 possible involvement in the pathogenesis of NEC intestinal damage. These studies indicated that the TLR pathway plays an important role in the pathogenesis of NEC.

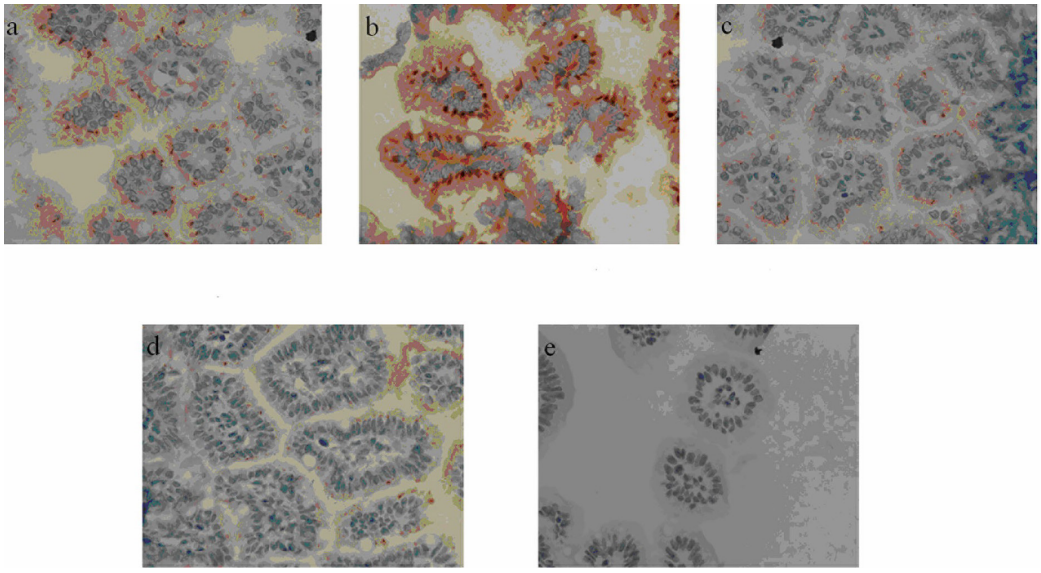

Figure 4. Immunohistochemistry can detect the expression level of NF-κB p65 in neonatal intestinal tissues of rats in each group (200X). There was no nuclear staining of NF-κB p65 in intestinal villi of Group E (**e**) rats. In groups C (**c**) and D (**d**), some nuclei stained can be seen. Nuclear staining increased significantly in group A (**a**), while Group B (**b**) showed a large nuclear staining.

In the treatment of NEC or inflammatory bowel disease, the usage of probiotics has become increasingly popular. A meta-analysis study strongly suggested that probiotics have protective effects (Ganguli and Walker, 2011). Currently, probiotics are thought to promote a balance in intestinal bacteria and strengthen bowel function by optimizing the microbial balance, competitively excluding pathogens, promoting mucus secretion, producing bacteriocins, strengthening barrier integrity, and promoting intestinal immunity maturation (Gareau et al., 2010). No evidence has shown that probiotic use leads to an increased incidence of sepsis (Deshpande et al., 2010). Some clinical studies have shown that the use of probiotics and rapid uptake of probiotic milk can help stabilize bacterial colonization and reduce the incidence and severity of NEC in high-risk preterm children (Gareau et al., 2010). However, some scholars remain cautious. *Bifidobacterium* species are Gram-positive anaerobic bacteria, which are advantageous normal intestinal flora in newborns. In many countries, bifidobacteria are one of the most widely used probiotics in formula milk and probiotic preparations (Szajewska et al., 2010). However, Gram-negative bacteria accounted for most NEC infections. LPS from Gram-negative bacteria induces an inflammatory cascade mainly caused by the TLR4 signal-

ing pathway; the cell wall of Gram-positive bacteria is primarily recognized by TLR2.

In this study, we found that in control groups of rat-milk-fed mice, the content of TLR2 and TLR4 in intestinal tissue was low. The expression of NK-κB was negative. These results indicate that under normal circumstances, because of the presence of the intestinal mechanical barrier and microbial barrier to the normal intestinal flora, direct contact with the intestinal pathogens was avoided, ensuring that the colonic mucosa cell TLR/NF-κB pathway was not activated by intestinal bacteria, thereby preventing an excessive inflammatory reaction, and these may play a protective role in the human body. Compared with the control group rats, which were fed rat's milk, the levels of TLR2, TLR4, and NF-κB p65 in intestinal tissue of the NEC model group rats were increased, indicating that LPS/TLR-mediated signaling pathways play an important role in the pathogenesis of NEC. In the bifidobacterium-intervention group, compared with the NEC model group, the levels of TLR2, TLR4, and NF-κB p65 decreased significantly, indicating that bifidobacteria can inhibit bacterial growth and reproduction, reduce the bacteria stimulation of intestinal mucosal cells TLR2 and TLR4, and thereby decrease the severity of NEC to some degree.

Conflicts of interest

The authors declare no conflict of interest.

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