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BRIEF ARTICLES

Effect of Lianshu preparation on lipopolysaccharide-induced diarrhea in rats

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Abstract

AIM: To investigate the effect of Lianshu preparation on lipopolysaccharide (LPS)-induced diarrhea in rats.

METHODS: A diarrhea model was established in Sprague Dawley rats *via* injection of 1 mL of 30 mg/kg LPS. A total of 40 rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group (n = 10 in each group). Their intestinal mucosal barrier and frequency of diarrhea were observed. Levels of glucose, serum Na^+, K^+, Cl^- and hematocrit, plasma nitrogen monoxide (NO), diamine oxidase (DAO), and D (-)-lactate were measured. The number of IgA+ plasma cells in small intestine was detected and SIgA levels in the intestinal fluid were measured. The antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia (10 mL/kg of 20% aqueous suspension). Acetaminophen (250 mg/kg, intragastric administration, *bid*) was used as a standard drug for comparison. Temperature was recorded 1 h before and 6 h after Brewer's yeast injection. Finally, small intestinal transmission in mice treated with Lianshu was detected after intraperitoneal injection of methyl prostigmin (2 mg/kg). Atropine (10 g/kg) was used as a control. The ink content in intestine was determined and the total length of intestine was measured.

RESULTS: The frequency of diarrhea was higher in LPS group than in LPS + Lianshu group and LPS + berberine group (36.70 ± 5.23 *vs* 28.50 ± 4.06 and 32.70 ± 9.30 respectively, P < 0.01), and lower in LPS + Lianshu group than in LPS + berberine group (P = 0.03). The levels of Na^+, glucose, Cl^-, K^+ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L *vs* 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L *vs* 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L *vs* 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L *vs* 4.32 ± 0.62 mmol/L respectively, P < 0.05). The level of hematocrit was lower in LPS + Lianshu group than in LPS + berberine group (0.50% ± 0.07% *vs* 0.59% ± 0.10% respectively, P < 0.05). The plasma levels of NO, DAO and D (-)-lactate were higher in LPS group than in normal group (79.74 ± 7.39 μmol/L *vs* 24.94 ± 3.38 μmol/L, 2.48 ± 0.42 μ/mL *vs* 0.82 ± 0.33 μ/mL, 5.63 ± 0.85 μg/mL *vs* 2.01 ± 0.32 μg/mL respectively, P < 0.01), and lower in LPS + Lianshu group than in LPS + berberine group (48.59 ± 4.70 μmol/L *vs* 51.56 ± 8.38 μmol/L, 1.43 ± 0.53 μmol/mL *vs* 1.81 ± 0.42 μmol/mL, 4.00 ± 0.54 μg/mL *vs* 4.88 ± 0.77 μg/mL respectively, P < 0.05). The morphology of the intestinal mucosa showed destroyed villi in LPS group and atrophied intestinal mucosa in other groups. The pathological intestinal mucosal changes were less in LPS + Lianshu group than in LPS group. The number of IgA+ plasma cells and amount of SIgA were higher in LPS + Lianshu group than in LPS group (1.16 ± 0.19/μm^2 *vs* 1.09 ± 0.28/μm^2, P = 0.026; 0.59 ± 0.12 mg/L *vs* 0.15 ± 0.19 mg/L respectively, P = 0.000). Lianshu had counteractive effects on yeast-induced pyrexia and enterokinesia in rats.

CONCLUSION: Lianshu preparation has therapeutic effects on LPS-induced diarrhea and enterokinesia in rats.

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Key words: Lianshu preparation; Lipopolysaccharide; Diarrhea; Nitrogen monoxide; D-lactate

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INTRODUCTION

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). Antibiotics developed in the 1940s have saved millions of people's life. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Antibiotic-resistant bacteria and side effects of antibiotics severely threaten the general welfare and health of people. Folk herbal drugs have been extensively studied in recent years for the treatment of acute diarrhea. This study was to investigate the effect of Lianshu preparation on LPS-induced diarrhea in rats.

MATERIALS AND METHODS

Animals

Eighty adult female and male Sprague-Dawley rats, weighing 230-280 g (Experimental Animal Breeding Center, Fudan University), and thirty 5-wk-old female and male Kunming mice, weighing 18-20 g, were used in this study following the guidelines of the Animal Care Committee of Institute of Chinese Medicine, Tongji University. The animals were housed at 22 ± 2℃ with a humidity of 55% ± 5% in a 12-h light/dark cycle for at least 1 wk prior to use, with free access to laboratory chow and tap water.

Lianshu preparation

Coptis chinensis, atractylodis, pulsatillae and portulacae, provided by Leiyunshang Company, were treated twice at 100℃ for 2 h, divided into 1:5:5:5 proportions, then treated at 100℃ for 1 h, filtered and concentrated to a relative density of 1.25 (60℃), and finally, desiccated. The effective constituents of Lianshu preparation were determined by Ley's Company (Shanghai, China). Lipopolysaccharide from *Escherichia coli* serotype 0128: B12 was purchased from Sigma Aldrich (St Louis, MO, USA). Nitrogen monoxide (NO) assay kit was provided by Jiancheng Bionic Research Center (Nanjing, China). Diamine oxidase (DAO) standard preparation, O-dianisidine, horseradish peroxidase, 1,5-pentanediamine-di-hydrochloride, D-lactate standard preparation, NAD- aminoacetic acid, and D-lactic dehydrogenase were purchased from Sigma Aldrich (St Louis, MO, USA). Anti-rat IgA mAb was purchased from Santa Crus Company (USA) and SIgA assay kit was purchased from Bethyl Labs Inc. (USA).

Detection of inhibitory effect of Lianshu preparation on LPS-induced diarrhea in rats

Rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group (n = 10 in each group). Before exposure to LPS, rats in normal and LPS groups were treated with distilled water, rats in LPS + Lianshu group were given Lianshu preparation (1.8 g/kg), rats in LPS + berberine group received berberine (0.2 g/kg) twice a day for 3 d. The rats were allowed to have water alone for 12 h. One hour after the last administration of drugs, rats in all groups apart from those in the normal group were treated with LPS (30 mg/kg).

Detection of diarrhea frequency before specimen collection

After treated with LPS, rats in each cage had a filter paper couch. Filter paper was changed once an hour for 4 h. The frequency of diarrhea was determined by counting the number of feces deposits on the filter paper.

Measurement of erythrocrit, blood glucose, and serum Na^+, K^+, Cl^- levels

Four hours later, 8 mL of blood was drawn from the abdominal aorta of each rat after anesthetized with 10 mg/kg intraperitoneal pentobarbital. Of the 8 mL blood, 2 mL was treated with heparin for erythrocrit analysis, 2 mL was centrifuged at 3000 × g for 15 min at 4℃. Blood glucose and serum Na^+, K^+, Cl^- levels were measured.

Measurement of plasma NO, DAO and D (-)-lactate levels

Four milliliters of blood was centrifuged at 3000 × g for 15 min at 4℃. Plasma was collected to measure NO at 550 nm as previously described[1]. DAO and D (-)-lactate levels were measured by spectrophotometry at 436 nm[2] and enzyme-linked ultraviolet spectrophotometry[3], respectively. The absorbance at 340 nm was recorded.

Observation of morphologic changes in intestinal mucosa

After humane killing, a 5-cm long section of ileum 10 cm below the Treitz ligament was removed, dissected longitudinally, fixed in 100 mL/L formalin, embedded in paraffin, stained with hematoxylin and eosin, and observed under a light microscope.

Detection of IgA+ plasma cells in rat small intestine

ABC immunohistochemistry staining of tissue paraffin sections was performed. The brown stained IgA + plasma cells were detected and analyzed using medical image analysis software (Taimeng Technology Co. Chengdu, China).

Determination of SIgA in small intestine

A small intestine segment about 5 cm long, 15 cm

Table 1 Serum electrolyte levels in different groups (mean ± SD)

Group	Sodium (mmol/L)	Glucose (mmol/L)	Chlorine (mmol/L)	Kalium (mmol/L)
Normal	143.46 ± 3.34	8.37 ± 1.06	102.03 ± 2.82[c]	5.37 ± 0.96
LPS	102.48 ± 3.67[b]	4.99 ± 1.23[b]	98.40 ± 1.42[a]	3.38 ± 0.29[b]
LPS + Lianshu	140.35 ± 3.19[d]	8.49 ± 1.84[d]	106.29 ± 4.41[b,d]	5.08 ± 0.66[d]
LPS + berberine	131.99 ± 4.86[b,d,f]	6.54 ± 2.30[a,e]	102.5 ± 1.39[d,e]	4.32 ± 0.62[b,d,e]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* LPS group; [e]$P < 0.05$, [f]$P < 0.01$ *vs* LPS + Lianshu group.

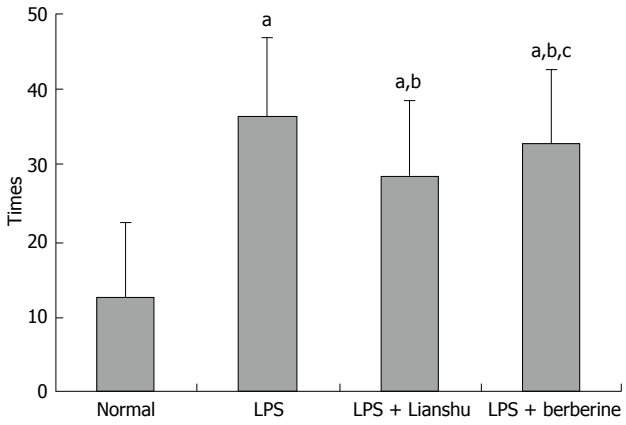

Figure 1 Frequency of diarrhea in different groups. [a]$P < 0.01$ *vs* normal group; [b]$P < 0.01$ *vs* LPS group; [c]$P < 0.05$ *vs* LPS + Lianshu group.

below the Treitz ligament, was removed. Intestinal fluid was collected by passing 5 mL PBS and 0.02% sodium into the small intestine. The first intestinal washing removed 85%-90% of total IgA, and the variability of samples was less than 5%. The washed out material was centrifuged at 3000 × *g* for 10 min at 4℃. The supernatant was harvested and stored at -70℃. Total IgA was determined by ELISA.

Assay of antipyretic activity of Lianshu preparation

Antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia. Forty rats were divided into 4 groups. Rats in control group were given sodium chloride (2 mL) and rats in experimental groups were given Lianshu preparation (1.8 g/kg), twice a day before yeast was injected. Acetaminophen (250 mg/kg, *bid*) was used as a standard drug to compare the antipyretic action of Lianshu preparation. One hour after the last administration of drugs, fever was induced by hypodermic injection of a 20% suspension of 10 mL/kg Brewer's yeast and rectal temperature was recorded 1 h before and 6 h after injection of Brewer's yeast. The antipyretic activity of Lianshu preparation in rats was assayed.

Detection of effect of Lianshu preparation on small intestine transmission in mice

Thirty Kunming mice, after 12 h fasting, were divided into sodium chloride group, Lianshu preparation group and atropine group ($n = 10$). Mice in the sodium chloride group received 0.5 mL sodium chloride by intragastric administration, twice a day for 1 d. Mice in the Lianshu preparation group received 1.8 g/kg Lianshu preparation, twice a day for 1 d. Mice in the atropine treatment group received 10 g/kg intraperitoneal atropine, twice a day for 1 d. All mice were given 2 mg/kg methyl prostigmin followed by 0.1 mL ink after 10 min, and killed by cervical vertebral dislocation with their belly cut open to collect intestines. The effect of Lianshu preparation on small intestinal transmission in mice was detected.

Statistical analysis

Data were expressed as mean ± SE and analyzed by analysis of variance and Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

General health of rats

No diarrhea and loose stools occurred in rats of the normal group. The fur of rats was smooth and the rats were active. Rats in the other groups that received LPS drank more water and had loose stools. The fur of rats was fluffy, and the rats were affected by lassitude. The respiration of rats was rapid.

Frequency of diarrhea after injection of LPS

The frequency of diarrhea in LPS group, LPS + Lianshu group and LPS + berberine group was increased compared to normal group (36.70 ± 5.23, 28.50 ± 4.06, 32.70 ± 9.30 *vs* 12.40 ± 3.20 respectively, $P < 0.01$), significantly increased in LPS + Lianshu group and LPS + berberine group compared with normal group ($P < 0.01$), and decreased in LPS + Lianshu group compared with LPS + berberine group ($P = 0.03$) (Figure 1).

Glucose, serum electrolyte and hematocrit levels in rats

The levels of glucose, Na^+, Cl^-, and K^+ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L *vs* 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L *vs* 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L *vs* 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L *vs* 4.32 ± 0.62 mmol/L respectively, $P < 0.05$), while the hematocrit level was lower in LPS + Lianshu group than in LPS + berberine group (0.50% ± 0.07% *vs* 0.59% ± 0.10%, $P < 0.05$, Table 1, Figure 2).

Plasma NO and DAO levels in rats

The plasma NO and DAO levels were higher in LPS group than in normal group ($P < 0.01$) and lower in LPS + Lianshu group than in LPS + berberine group ($P < 0.05$, Table 2).

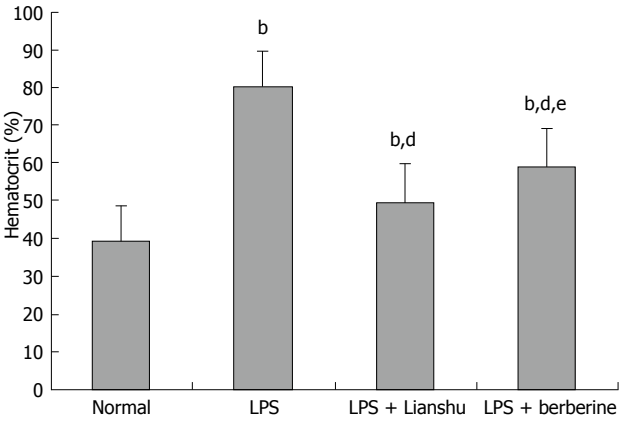

Figure 2 Hematocrit in different groups. $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.01$ *vs* LPS group; $^{e}P < 0.05$ *vs* LPS + Lianshu group.

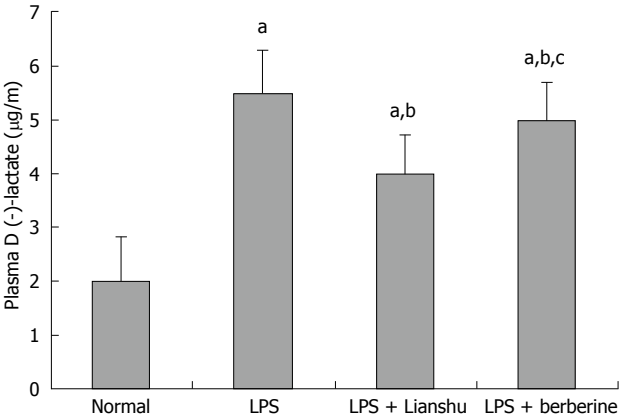

Figure 3 Comparison of plasma D (-)-lactate in different groups. $^{a}P < 0.01$ *vs* normal group; $^{b}P < 0.01$ *vs* LPS group; $^{c}P < 0.05$ *vs* LPS + Lianshu group.

Plasma D (-)-lactate levels in rats

Plasma D (-)-lactate level was higher in LPS group, LPS + Lianshu group and LPS + berberine group than in normal group (5.36 ± 0.85, 4.00 ± 0.54, 4.88 ± 0.77 *vs* 2.01 ± 0.32 respectively, $P < 0.01$), and lower in LPS + Lianshu group, LPS + berberine group than in LPS group and LPS + berberine group ($P < 0.01$, Figure 3).

Morphological changes in intestinal mucosa

The villi of intestinal mucosa maintained their integrity and the shape of epithelial cells remained unchanged in the normal group. The villi were destroyed in the LPS group. Intestinal mucosal atrophy and inflammatory reaction were detectable in the other groups (Figure 4).

Staining and number of IgA+ plasma cells in intestinal mucosa

IgA+ cells, round or elliptical in shape, were distributed in the intestinal villi, lamina propria and intestinal glands. The endochylema was stained brown (Figure 5).

Number of IgA+ cells and SIgA in intestinal fluid

The number of IgA+ cells and amount of SIgA in intestinal fluid were less in LPS group than in normal group ($P < 0.01$) and higher in LPS + Lianshu group than in LPS + berberine group ($P = 0.05$, Table 3).

Table 2 Plasma NO and DAO levels in different groups (mean ± SD)

Group	No (μmol/L)	DAO (μmol/mL)
Normal	24.94 ± 3.38	0.82 ± 0.33
LPS	79.74 ± 7.39^{b}	2.48 ± 0.42^{b}
LPS + Lianshu	48.59 ± 4.70^{b,d}	1.43 ± 0.53^{b,d}
LPS + berberine	51.56 ± 8.38^{b,d,f}	1.81 ± 0.42^{b,d,e}

$^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.01$ *vs* LPS group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* LPS + Lianshu group.

Table 3 Number of IgA+ cells and SIgA in intestinal fluid of different groups (mean ± SD)

Group	IgA+ cells (/μm^2)	SIgA (mg/L)
Normal	0.94 ± 0.21	0.50 ± 0.09
LPS	0.73 ± 0.22^{a}	0.22 ± 0.66^{b}
LPS + Lianshu	1.16 ± 0.19^{b,d}	0.59 ± 0.12^{d}
LPS + berberine	1.09 ± 0.28^{a,d,e}	0.15 ± 0.19^{b,c,e}

$^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* LPS group; $^{e}P < 0.05$ *vs* LPS + Lianshu group.

Counteractive effect of Lianshu preparation on yeast-induced pyrexia in rats

The temperature of rats in yeast group and sodium chloride group was similar 1 h after treatment with Lianshu preparation and higher in yeast group than in sodium chloride group 2-5 h after treatment with Lianshu preparation ($P < 0.05$). The temperature was not significantly changed in yeast + Lianshu group compared to yeast group 1 h or 2 h after treatment with Lianshu preparation. The temperature in yeast + Lianshu group decreased 3 h after treatment with Lianshu preparation ($P = 0.025$), and was almost identical to that in the normal group 4 h after treatment with Lianshu preparation. The temperature was lower in yeast + acetaminophen group than in the sodium chloride group 2 h after treatment with Lianshu preparation. Yeast + acetaminophen exhibited their effect 1 h earlier than yeast + Lianshu preparation (Table 4).

Counteractive effect of neostigmine on enterokinesia in mice

The rate of small intestinal peristalsis for mice after treatment with Lianshu preparation and atropine was different from that in mice after treatment with sodium chloride (55.20% ± 10.16% and 39.96% ± 8.57% *vs* 65.10% ± 10.93% respectively, $P < 0.05$, Figure 6).

DISCUSSION

A group of Japanese workers reported that parenteral LPS can lead to proliferation of gastrointestinal luminal bacteria in mice[4]. Subsequent work established that this enhancement of bacterial growth is secondary to

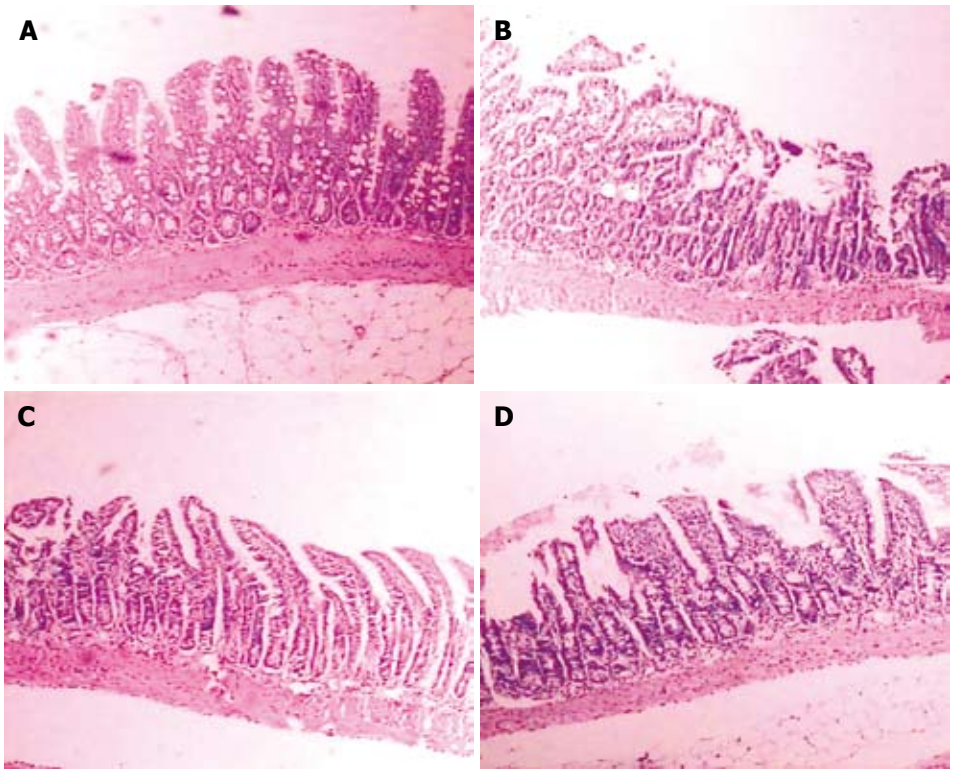

Figure 4 Optical microscopic observation of ileum mucosa of rats (HE, × 100). A:Normal group; B: LPS group; C: LPS + Lianshu group; D: LPS + berberine group.

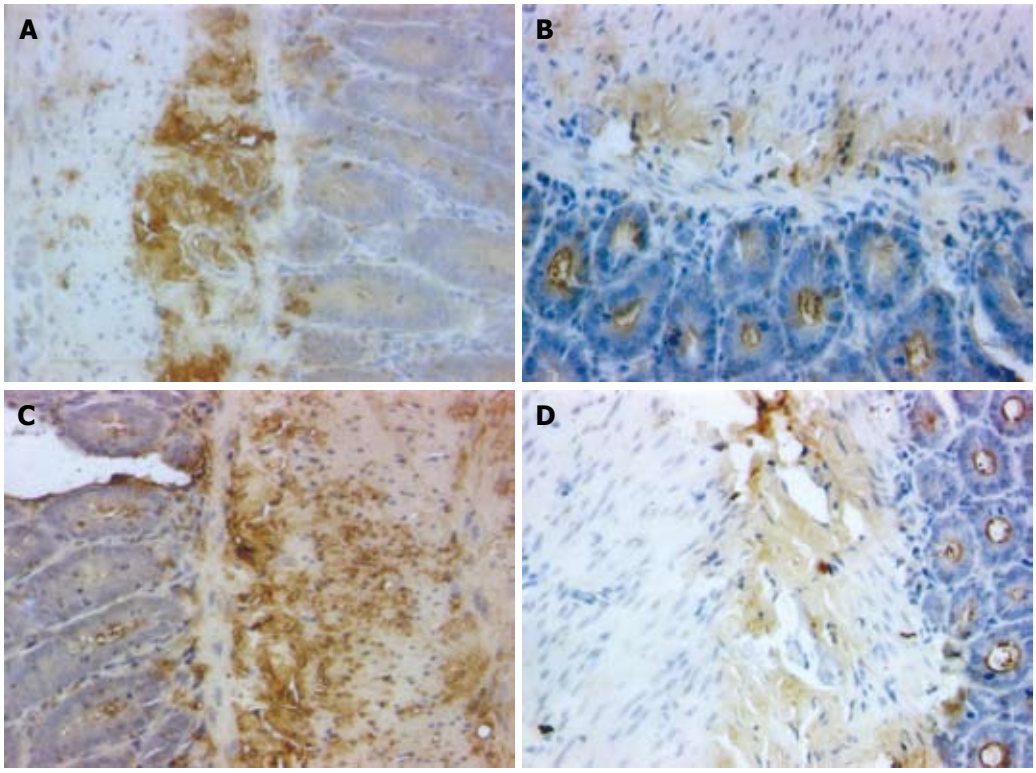

Figure 5 Staining and number of IgA+ plasma cells in intestinal mucosa (× 200). A: Normal group; B: LPS group; C: LPS + LianShu group; D: LPS + berberine group.

fluid exudation, since the growth of bacteria could be suppressed by enteral unabsorbable antibiotics like neomycin[5]. Subcutaneous LPS from Gram-negative bacteria induces intestinal diarrhea characterized by destruction of the intestinal mucosa barricade[6-9].

In the present study, the frequency of diarrhea and gastrointestinal transit (GIT) were detected, and the levels of NO, DAO and D (-)-lactate were measured in mice after treatment with intraperitoneal LPS. Lianshu preparation could effectively prevent diarrhea by inhibiting enterokinesia. Generally, NO can protect and repair gastrointestinal mucosa. Endogenous NO has therapeutic effects on hypoxia, inflammation and damage. However, excess NO is probably one of the most important mediators that induce blood poisoning, septic shock and multi-organ dysfunction[10]. When stimulated by LPS, NO is excessively expressed and induces multi-organ functional lesions in stomach and intestine[11-13].

Table 4 Counteractive effects of yeast on pyrexia in different groups (Δ℃) (mean ± SD)

Groups	1 h	2 h	3 h	4 h	5 h	6 h
Normal	0.58 ± 0.28	0.24 ± 0.71	0.20 ± 0.83	0.47 ± 0.30	0.17 ± 0.51	0.27 ± 0.65
Yeast	0.14 ± 0.94	0.75 ± 0.28[b]	1.06 ± 0.28[b]	1.16 ± 0.39[b]	1.69 ± 0.43[b]	1.46 ± 0.59[b]
Yeast + Lianshu	0.30 ± 0.38	0.58 ± 0.38	0.44 ± 0.56[c]	0.42 ± 0.29[d]	1.01 ± 0.43[c]	0.50 ± 0.44[d]
Yeast + acetaminophen	0.11 ± 0.73	0.21 ± 0.39[c]	0.37 ± 0.53[c]	0.66 ± 0.50[d]	0.74 ± 0.97[a,d]	0.53 ± 0.98[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* yeast group.

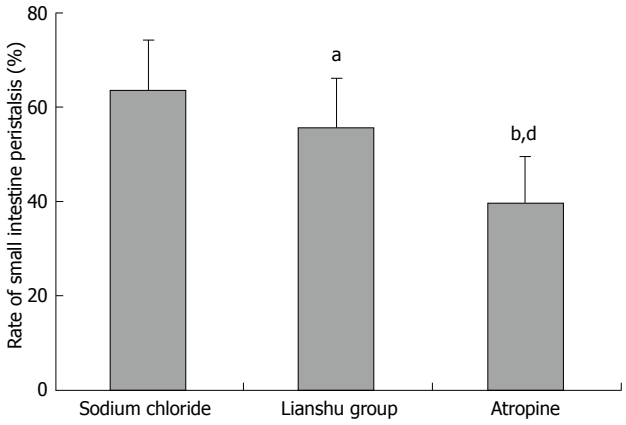

Figure 6 Counteractive effects of neostigmine on enterokinesia of mice. [a]$P < 0.05$, [b]$P < 0.01$ *vs* sodium chloride group; [d]$P < 0.01$ *vs* Lianshu group.

In this study, Lianshu preparation prevented enteritis necroticans by inhibiting the production of excess NO. DAO is an ideal index of intestinal mucosal structure and function. The activity of DAO is closely related with villi, nucleic acid and protein synthesis in mucosal cells. When intestinal mucosal epidermis is damaged, DAO is released into blood, indicating that DAO in blood reflects the destruction of the intestinal mucosal epithelial cell layer and intestinal mucosa barricade[14,15]. In our study, DAO was localized mostly in the epithelial cell layer of mucous membrane as previously described[16], and Lianshu preparation could effectively decrease DAO induced by LPS. However, its mechanism of action remains unclear. In addition, the function of the intestinal mucosa barricade could also be evaluated by measuring D (-)-lactate in plasma[17-19]. D (-)-lactate is a product of bacterial metabolism and schizolysis. However, mammals cannot produce D (-)-lactate[20]. D (-)-lactate cumulation in plasma reflects membrane permeability and barrier function of the intestinal mucosa[21,22]. In our study, Lianshu preparation could reduce D (-)-lactate in plasma ($P < 0.01$) by protecting the barrier function of the intestinal mucosal and suppressing the overgrowth of harmful bacteria in the intestinal tract.

IgA is the main immunoglobulin in the mucosal immune system[23]. Polymeric IgA antibodies produced by plasma cells in the lamina propria of the intestinal tract bind to polymeric immunoglobulin receptors at the base of the epithelium. The complex of IgA and polymeric immunoglobulin receptors undergoes endocytosis and vesicular transport to the apical surface of enterocytes, and is secreted into the lumen. Generally, plasma cells are derived from B cells. It was reported that B cells are able to switch from IgM expression to IgA expression by Th2 cytokine activity in Peyer's patches of the lamina propria and mesenteric lymph nodes, and then return to lamina propria of the intestinal tract *via* systemic circulation[24-26]. LPS, a mouse B-cell mitogen, induces B cell multiplication[26] and differentiation into plasma cells. LPS also activates macrophages to promote the production of cytokines such as interleukin (IL)-1, IL-6 and nitric oxide, which might regulate immunoglobulin production. Therefore IgA inhibits diarrhea caused by viruses[27-29]. Moreover, LPS might activate IgA production in the intestine[30]. However, in our study, the number of IgA+ cells and SIgA in the intestinal fluid was less in the LPS group than in normal group, and greater in the LPS + Lianshu group than in the LPS group, suggesting that high dose LPS leads to severe destruction of intestinal mucosa.

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COMMENTS

Background

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). It is the important task of modern medicines to control infectious diarrhea. Antibiotics developed in the 1940s have saved millions of people's lives. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Folk herbal drugs have been studied for the treatment of acute diarrhea.

Research frontiers

The method to treat diarrhea is mainly to kill bacteria or viruses. Lianshu preparation could destroy bacteria, prevent diarrhea, and protect the intestinal mucosa.

Innovations and breakthroughs

Lianshu preparation, comprised of non-antibiotic botanic components, has been used in the treatment of infectious diarrhea with a satisfactory effect. Lianshu preparation could effectively prevent diarrhea, relieve electrolyte disturbances and protect immune barriers. Its effect is better than berberine. In addition, Lianshu preparation has counteractive effects on pyrexia.

Applications

Lianshu preparation can be used as a natural medicine in treatment of infectious diarrhea with pyrexia. Chinese herbal drugs are effective against both chronic and acute diseases.

Peer review

The authors confirmed, in their study, that Lianshu preparation was effective against LPS-induced diarrhea, its effect was better than berberine, suggesting that it can be used as a natural medicine in treatment of infectious diarrhea.

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