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Original Article

Effects of Rifaximin on visceral sensitivity of rats with diarrhea induced by folium sennae

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Abstract: Objective: To observe the therapeutic action of Rifaximin on rats with diarrhea and visceral sensitivity. Methods: The diarrhea rat models were established by gastric gavage of folium sennae and shackled hindlimbs. Diarrhea-index was used to assess outcomes, abdominal withdrawal reflex (AWR) scores were observed in colorectal distention (CRD) experiment. Intestinal mucosa was observed by hematoxylin-eosin (HE) staining. Brain-derived neurotrophic factor (BDNF) expression in colon was detected by immunostaining and Western-blot methods. Results: Compared with the control group, intestinal mucosa has not shown apparent injury in model group by HE staining. Compared with the model group, the diarrhea-index and AWR scores in drug administration group were both reduced ($P<0.05$). In addition, BDNF expression in colon was significantly reduced in drug administration group by immunohistochemistry and Western blot analysis ($P<0.01$). Conclusion: Rifaximin has antidiarrheal effect on the rats with diarrhea caused by folium sennae and shackled hindlimbs and could decrease visceral hypersensitivity, which was related with the reduction in BDNF expression in colon.

Keywords: Rifaximin, diarrhea rats, visceral hypersensitivity, brain-derived neurotrophic factor (BDNF), abdominal withdrawal reflex (AWR)

Introduction

Abdominal pain and diarrhea are common symptoms of functional gastrointestinal disorders (FGIDs). Negative emotions may easily cause FGIDs and worsen abdominal pain and diarrhea. Hypersensitivity in visceral has been regarded as one major pathophysiologic feature of FGIDs, such as irritable bowel syndrome [1, 2].

Rifaximin is the semi-synthetic derivative of rifamycin. Rifaximin possesses an additional pyridoimidazole ring, which makes it minimally absorbable in gastrointestinal tract. It is commonly used for the treatment of acute or chronic intestinal infection or other gastrointestinal infectious diseases with less influence on the normal intestinal flora [3-6]. Rifaximin can quickly release spastic abdominal pain, headache, nausea and vomiting. However, its influence on intestinal sensitivity and relevant mechanisms is rarely reported.

BDNF, which is widely distributed in nervous system, plays an important role in differentiation, growth and damage restoring, and can maintain the normal function of sensory nerve and its neural pathway [7, 8]. The increased level may result in various abnormal feelings related to pains such as chronic pain, inflammatory pain, visceral pain and high sensitivity [9]. However, its influence on intestinal sensitivity in rats with diarrhea has not been reported.

The aims of this study were to build a diarrhea rat model accompanied with emotion changes by giving folium senna and restrainting their hindlimbs. The effects of rifaximin on diarrhea [10, 11] and visceral sensitivity that are caused by liver-spleen disharmony related to negative emotions were observed. The relationship between BDNF and intestinal hypersensitivity of diarrhea rates was also observed.

Materials and methods

Experimental animals

Adult male Wistar rats of clean grade obtained from Dalian Medical University Lab. Animal Center (SCXK (Liao) 2008-0002). The weight of each rat was 170-190 g.

Table 1. Effect of Rifaximin on loose stool rate, average loose stool degree and diarrhea index of rats ($\bar{X}\pm s$)

Group	Number of animals	Loose stool rate	Average loose stool degree	Diarrhea index
Control group	8	0	0	0
Model group	8	0.94±0.06	2.73±0.27	2.57±0.26
Pinaverium Bromide	8	0.60±0.11**	2.29±0.30	1.38±0.43**
Rifaximin (40 mg/kg)	8	0.76±0.09**	2.37±0.32	1.80±0.32**
Rifaximin (80 mg/kg)	8	0.46±0.08**	1.74±0.18**	0.79±0.10**
Rifaximin (160 mg/kg)	8	0.09±0.10**	1.00±1.00**	0.16±0.21**

**P<0.01 vs. model group.

Drugs

Folium senna (bought from Haiwang Xingchen Drugstore) was soaked in a defined amount of distilled water for 30 min, boiled for 10 min, and filtered by a two-tier gauze. Then the filtrate was compressed and 0.2 g/ml solution was made from the crude drug content. Rifaximin was purchased from Chengdu Yuyang High-Tech Development Co., Ltd. (Lot No. 100501). Pinaverium Bromide was purchased from Abbott Healthcare SAS. (Lot No. H20120127).

Reagent and instrument

Brain-derived neurotrophic factor (BDNF) rabbit anti-rat antibody was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. LTD; UltraSensitiveTM S-P Kit was purchased from Fujian MAIXIN Biotechnology Co. LTD; 8#FR catheter was purchased from Jiangsu Conod Medical Co. Ltd. All solvents used in this study were analytical grade.

Groups and drug administration

The 48 Wistar rats were randomly divided into 6 groups, one normal group (normal saline control), one model group, one positive control group (Pinaverium Bromide, 20 mg/kg), and experimental groups with different rifaximin dosages (40 mg/kg, 80 mg/kg, 160 mg/kg). 8 rats were included in every group. All the rats stayed in laboratory for a week before the experiment.

Model replication

The model was built according to literatures [12, 13] and being improved. The model group was given 0.2 g/ml folium senna apozem, at 10 ml/kg by gavage once a day. One hour after gavage, their hind limbs were tied by rough cotton ropes for 2 hours. Drugs and bandage-stress were continuously given for 14 days. Since the fifteenth day, the rats of positive control group and the experimental groups were given the corresponding medicines for 14 days. The animals were raised in single dry experimental cages, with filter paper on the cage floor.

Diarrhea criteria, diarrhea rate and diarrhea-index [14, 15]

Two hours after the last gavage, the characters of rat feces were collected and observed within 3 hours, and loose or dry stools were judged by whether there was stain on the filter paper. Number of feces: one pellet or one pile stool was judged as one feces. Loose stool rate was determined as the ratio of loose stools to total stools for each animal. The degree of loose stools of each animal was judged by the stain diameter on the filter paper: first degree <1 cm, second degree 1~2 cm, third degree 2~3 cm, and fourth degree >3 cm. The diarrhea rate and diarrhea index were calculated as follows:

Average loose stool degree = total loose stools degrees/times

Diarrhea criteria = number of diarrhea animals/number of animals in the group

Diarrhea-index = Loose stools rate × average loose stools degree

AWR scores

Intestinal tract sensitivity determination [16] by colorectal distention (CRD) test, grade abdominal withdrawal reflex (AWR), Score standards are as follows:

0 The rats had stable emotion when given CRD stimulation, but had no behavioral response to distention; 1 The rats were in emotional instability, twisting the head; 2 The back and abdominal muscles of rats were contracted lightly but their belly weren't lifted; 3 The back and abdominal muscles of rats were contracted strongly and their belly was lifted off the floor; 4 The belly muscles of rats were contracted more strongly, their back was arched, and their belly, pelvis and perineum were lifted off the floor.

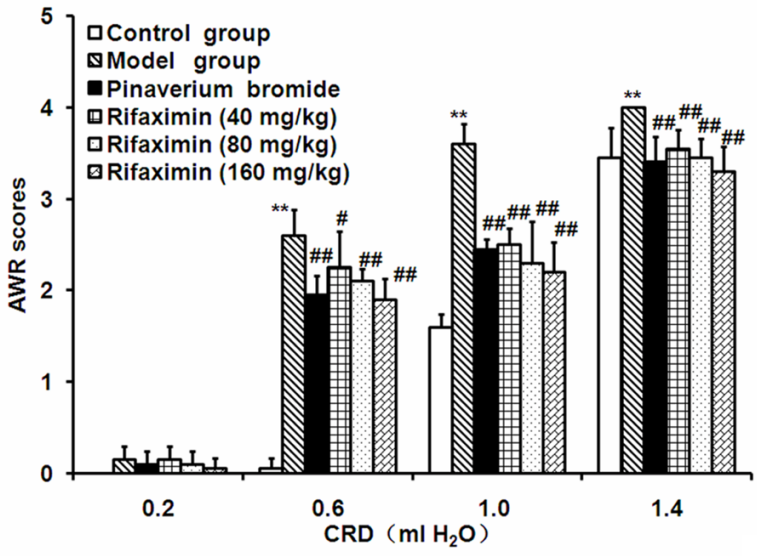

Figure 1. Effect of rifaximin on AWR scores of diarrhea rats. AWR scores analyses were performed to investigate the intestinal sensitivity in each group. When volume of H_2O was 0.6 ml **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. model group; When volume of H_2O was 1.0 ml **P<0.01 vs. control group; ##P<0.01 vs. model group; When volume of H_2O was 1.4 ml **P<0.01 vs. control group; ##P<0.01 vs. model group.

Eighteen hours before the last gavage, the rats were fed nothing but water. One hour after administration, the rats were put into the fixator and were restrained to turn back. An 8F catheter covered with Vaseline was inserted into the anus, leaving 1 cm between the gasbag-end and anus. The catheter and the rat tail were fixed with adhesive tapes. Warm normal saline (37-38°C) was injected into the gasbag of catheter in 20 min later, the volume was gradually increased by 0.2, 0.6, 1.0 and 1.4 ml (each volume was lasted for 20 s, then had a break for 3 mins). Two observers conducted AWR grading separately. If AWR grade was 4, no more volume experiment would be done. 30 minute's break was had between two CRDs, AWR grading was repeated twice on each rat for an average grade.

Staining process of HE

The rats were decapitated, and a 1 cm colon (5 cm above anus) was taken out and cleaned with 5% formaldehyde. Then each colon segment was fixed in 10% formalin, routinely dehydrated, embedded into paraffin, continuously sliced, deparaffinized and rehydrated. Then it was stained by haematoxylin and eosin. Then hematoxylin was dehydrated in 70%, 90%, 95% ethanol, cleared in xylene, mounted in Permount or Histoclad. Finally, morphological changes were observed under microscope.

Immunohistochemistry (IHC) of BDNF expression

The same colon segment was taken by using the method above, fixed with 10% neutral formalin, embedded in paraffin after routine dehydration, and sliced. Then each sample was operated by using kit method, and stained by IHC.

Western-blot analysis of BDNF expression

After the tissue parts mentioned above were taken, the content of the intestines was washed by saline solution, quick-freezed in a liquid nitrogen tank, and kept in a fridge at -80°C. Then cell lysis buffer was added and they were homogenate and centrifuged at 4°C and 12000 g for 15 min. The protein content of the supernatant was determined by using the BCA protein assay kit. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer was added at 95°C for 10 min in order to make it degenerated. Depending on the different antibodies, equivalent total protein was loaded in each lane of SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was sealed up in 5% skimmed milk powder at 37°C for 1.5 h. Membranes were incubated with the BDNF and β-actin primary antibodies at 4°C overnight. And a corresponding secondary antibody was incubated for 2 h at room temperature. After washing, BeyoECL Plus kit proteins were developed to take photographs and undergo analysis by gel imaging system. β-actin as internal reference, the gray of target protein and internal reference protein ratio express the relative expression amount of protein.

Statistical analysis

Data were analyzed with SPSS 11.5 (SPSS Inc., USA), and the significance level was set at P<0.05. The results of loose stool rate, average loose stool degree, diarrhea index, AWR grad-

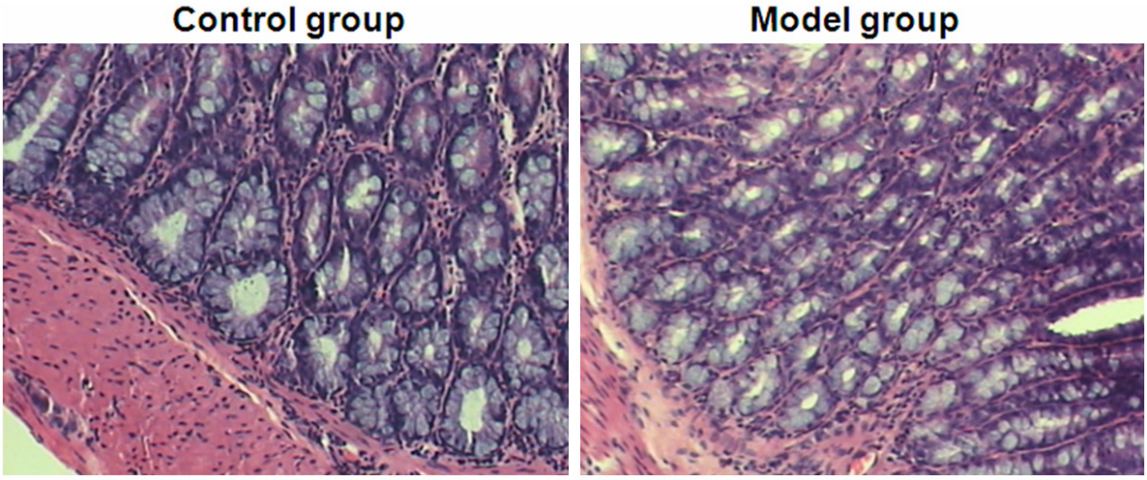

Figure 2. HE staining of colon tissue in control and diarrhea groups. Each group has a complete colon structure and aligned cells, and there are no evident abnormal changes.

ing and Western-blot analysis of BDNF protein expressions were both expressed as mean ± SD (standard deviation) ($\bar{X}\pm s$). Inter-group comparison was tested by one-way ANOVA.

Results

Diarrhea rate and diarrhea index

The faeces were brown, soft and formless pellets or even with mucus in them in the rats of the model group, and their diarrhea rate was 100%. While in the normal control group, the rats defecated granular feces, without stain on filter paper, so the diarrhea rate was 0. The three experimental groups and the positive control group had a similar situation as the model group before administration, and the diarrhea rate was 100%. After being administered with corresponding drugs for 14 days, the diarrhea rate and diarrhea index were significantly decreased ($P<0.01$) (**Table 1**).

Intestinal tract sensibility testing

When CRD rectal expansion volume was in 0.2 ml, 0.6 ml, 1.0 ml and 1.4 ml, AWR scores of different groups were shown in **Figure 1**. The intestinal sensitivity of the model group was obviously higher than that of the control group, and the intestinal sensitivity threshold was lower than that of the control group ($P<0.05$). After medicine intervention, the intestinal sensitivities of the positive control group and the experimental groups were lower than that of the model group ($P<0.05$), but the intestinal sensitivity threshold had no evident change ($P>0.05$).

Intestinal HE dyeing

Rat colon HE dyeing results are shown in **Figure 2**. Each group had complete colon structure and aligned cells, and there was no evident abnormal changes, such as erosion or ulcer. In the model group, mild swelling could occasionally be seen under mucous membrane, and local inflammatory infiltrated, goblet cells increased

BDNF expression by IHC

Rat intestinal BDNE IHC results were shown in **Figure 3**. BDNF was mainly expressed in intestinal mucosa epithelial cells. In colon, BDNF expression was higher in the model group than that in the blank control group. After drug intervention, BDNF expression was decreased evidently in the positive control group and the three experimental groups in comparison with the model group.

BDNF expressions by Western-blot

Western blotting results (**Figure 4**) indicated that, BDNF in rat colon tissues of the model group increased evidently in comparison with the blank control group ($P<0.01$). After drug intervention, BDNF expression decreased evidently in the positive control group and the three experimental groups in comparison with the model group. The decrease of BDNF expres-

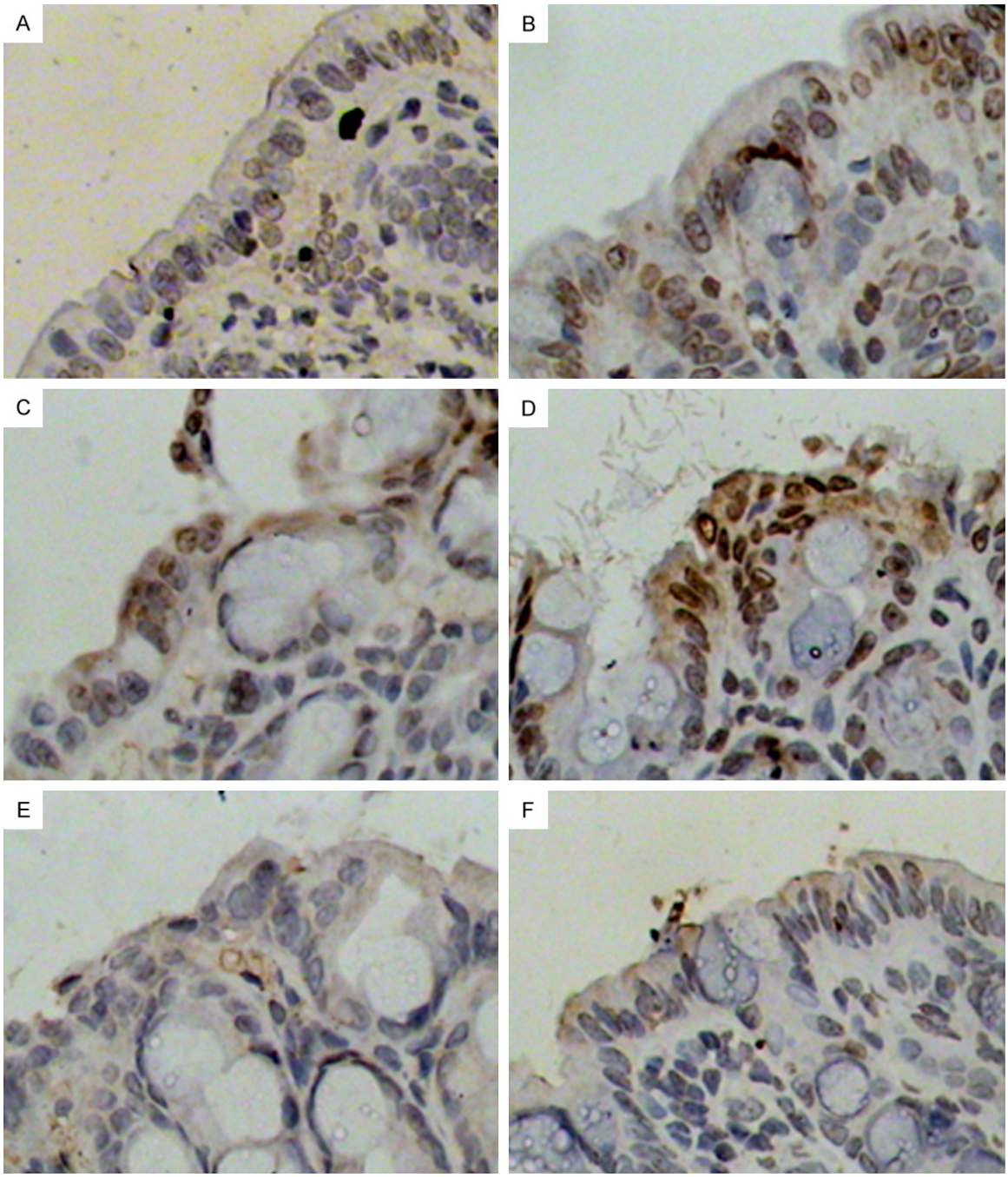

Figure 3. Immunohistochemical staining of BDNF on colon tissue sections in rats (400×). A. Control group; B. Model group; C. Pinaverium bromide group; D. Rifaximin group (40 mg/kg); E. Rifaximin group (80 mg/kg); F. Rifaximin group (160 mg/kg). The expression level of BDNF was increased in the model group (diarrhea rates). The BDNF expression was decreased evidently in pinaverium bromide group and rifaximin groups (80 mg/kg, 160 mg/kg).

sion of the medium dosage and high dosage groups were significantly different from the model group ($P<0.01$).

Discussion

Traditional Chinese Medicine holds that diarrhea can be caused by exogenous evils such as summer-heat and damp, raw and hot; or by dirty diet, dyspepsia; or by emotional disorder. Folium senna contains anthraquinone glycoside, which can improve colon tension and restrain colon and rectum from absorbing water and electrolyte, thus detaining water in enteric cavity, increasing power propulsion of large intestine, and inducing diarrhea [17]. Emotion

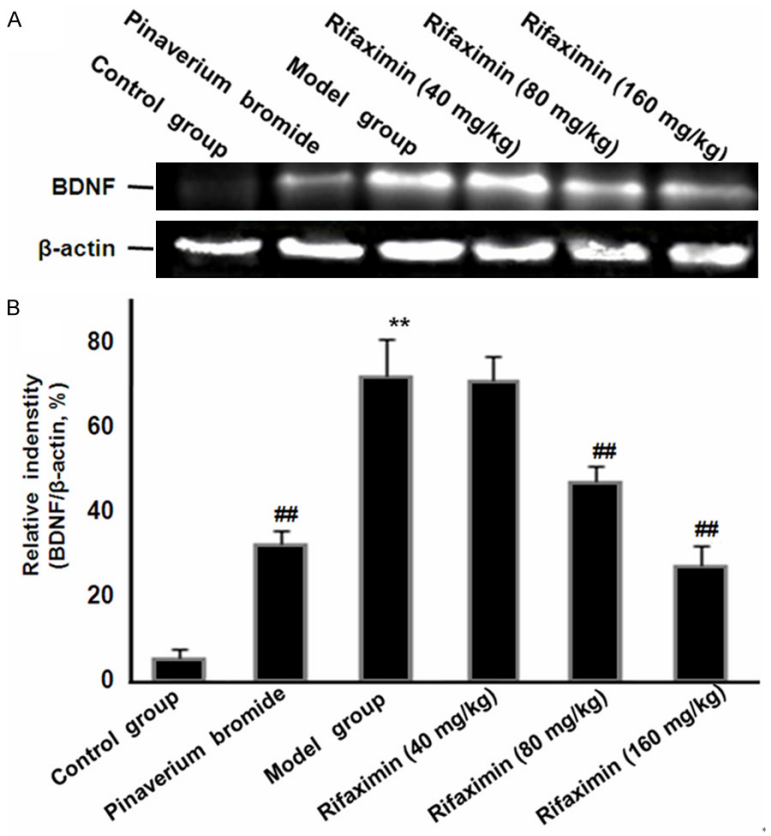

Figure 4. Effects of rifaximin on BDNF protein expression in colon tissues of rats. Compared with normal control group, the protein contents of BDNF were significantly increased in diarrhea rats (**P<0.01). Compared with diarrhea rats, the protein contents of BDNF were significantly decreased (##P<0.01) by Pinaverium bromide and rifaximin at the dosage of 80 mg/kg and 160 mg/kg.

stress is a type of complicated reaction to neurohumoral control, which may lead to FGIDs and cause abnormal defecation [18]. Reportedly, restraint stress can evidently increase the susceptibility of rat viscera [19].

Small-dosage folium senna gavage in combination with restraint stress will lead to spleen stomach disorder and diarrhea symptoms; limbe constraint will enrage the experimental animals, increase loose stools, and visceral hypersensitivity in the rats of model group. Meanwhile, histological observation reveals that the model group had no obvious organic changes in intestinal mucosa shape. These changes accord with the changes of epidemiological characteristics and pathophysiologic characteristics of clinical gastrointestinal disorder in the diarrhea of irritable bowel syndrome (D-IBS).

The results in this study indicated that rifaximin could efficiently decrease the diarrhea index of diarrhea rats, which proved that rifaximin had anti-diarrhea effect on the animals. In the CRD experiment, Visceral pain threshold of rats were decreased and visceral sensibility were increased in model group. After rifaximin intervention, compared with the model group, AWR score was decreased, indicating that rifaximin could reduce the visceral hypersensitivity of rats. The results could provide experimental evidence of rifaximin would be effective for treatment of the patients with the diarrhea of irritable bowel syndrome.

IHC observation and quantitative Western-blot analysis showed that BDNF protein expression increased evidently in colon of the rats in model group, which provided more evidence for the relativity between abnormal BDNF increase and intestinal high sensitivity. The results were similar to the FGIDs, such as IBS patients, BDNF expression in intestine increased significantly, which had a close relation to the increase of its intestinal tract sensitivity [20]. After intervention of rifaximin, rats' colon mucosa BDNF protein expression decreased, indicating that rifaximin could decrease rat intestinal sensitivity by reducing colon mucosa BDNF expression, which helped to relieve pain. But more research was needed to do in order to discuss the detailed mode of action and the related effective accesses.

Disclosure of conflict of interest

None.

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