Pakistan J. Zool., vol. 51(1), pp 57-65, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.1.57.65

The Effects of Wumei Pill on TLRs/NF-kB Signaling Pathway in Rats with Diarrhea-Predominant Irritable Bowel Syndrome

Xiaojie Ding, Xiling Sun, Zuien Wang, Qiusheng Zheng, Xiaofei Yu, Wenjin Hao, Kejun Wang, Wenjuan Xu and Zhengping Dong*

Department of Integrated Traditional Chinese and Western Medicine, Binzhou Medical University, Yantai, 264003, China

ABSTRACT

The aim of the present study was to investigate the effects of Wumei Pill on the TLR4/9-NF-kB signaling pathway in rats with diarrhea-predominant irritable bowel syndrome (IBS-D). The model was established by means of acetic acid enema plus restraint stress. The rat fecal moisture content, fecal trait score, and abdominal wall withdrawal reflex (AWR) score were used to determine whether the model was successful. The effects of Wumei Pill on the TLR4/9-NF-kB signaling pathway rats with IBS-D was observed by testing the contents of serum level of tumour necrosis factor-α (TNF-a), interleukin-6 (IL-6), lipopolysaccharide (LPS) as well as colonic mucosal tissue TLR4/9 and NF-kB. We got the following results: (1) The rat IBS-D model was successfully prepared; (2) Wumei Pill could reduce the contents of serum TNF-α, IL-6 and LPS in rats($P<0.05$), and alleviate the inflammatory reaction and accumulation of bacterial endotoxins; (3) Wumei Pill could reduce the transcriptional level of TLR4/9mRNA, to decrease TLR4/9 protein synthesis and expression. So we concluded that Wumei Pill probably reduce the release of pro-inflammatory cytokines and the bacterial endotoxins by blocking the transmission of TLR4/9-NF-kB signaling pathway so that Wumei Pill has a significant therapeutic effect on IBS-D.

Article Information
Received 12 July 2018
Revised 29 August 2018
Accepted 21 September 2018
Available online 15 November 2018

Authors' Contribution
XD, ZD, XS, QZ and ZW designed the study. XD, ZD, XY, WH and KW performed the experimental work and analyzed the data. WX helped in microscopic examinations. XD and ZD wrote the article.

Key words
Wumei pill, IBS-D, TLR4/9-NF-kB signaling pathway, Inflammatory factor, LPS.

INTRODUCTION

Diarrhea-predominant irritable bowel syndrome (IBS-D) is an irritable bowel syndrome with diarrhea as the main clinical manifestation. At present, IBS-D is considered to be the result of a combination of multiple pathogenesis mechanisms (Tanaka *et al*., 2011; Ge and Xu, 2013; Zhang and Song, 2014). IBS-D is mainly accompanied by the low-grade inflammatory reaction (Liebregts *et al*., 2007). TLRs/NF-kB signaling pathway plays a significantly important role in the occurrence and development of inflammation (He and Huang, 2013). Wumei Pill is an effective prescription for the treatment of chronic diarrhea. Clinical practices and studies have shown that Wumei Pills have a very good therapeutic effect on IBS-D (Ni and Sun, 2014; Luo *et al*., 2016). The experimental study has shown that Wumei Pill can restrain the transmission of TLR4/NF-kB signaling pathway and the abnormal activation of TLR9/MyD88/NF-kBp65 signaling pathway (Yan, 2012; Li *et al*., 2016). In this study, the action mechanism of Wumei Pills on IBS-D was investigated from the perspective of the regulation and controlling of TLR4/9-NF-kB signaling pathway.

* Corresponding author: dingxiaojie0604@sina.cn
0030-9923/2019/0001-0057 $ 9.00/0

MATERIALS AND METHODS

Experimental instruments and medicines

Wumei Pills are manufactured by Kunming Chinese Medicine Co., Ltd. (GYZZ No. Z53020892) and probiotics are provided by US SWANSON Health Foods (60 capsules/bottle). The ELISA kits tumour necrosis factor -α(TNF-α), interleukin-6 (IL-6) and lipopolysaccharide (LPS) were purchased from Shanghai Blue Gene Biotech Co., Ltd. Test consumables include 10% chloralic hydrate,4% acetic acid (Tianjin Bodi Chemical Co., Ltd.), capillary glass tubes, gavage needles and catheter with balloon (Germany Braud). Instruments include the full-automatic tissue dehydrator (Tianjin Aihua Medical Instrument Co., Ltd.), slicing machine (German SLEE), CX40 optical microscope (Japan Olympus), DYY-6C electrophoresis apparatus (Bejing Liuyi Instruments Co., Ltd.) and DYYm40B water-bath electrophoresis transfer tank (Beijing Liuyi Instruments Co., Ltd.).

Experimental animals

SPF-level SD rats, male, 32 in total, 3-4 weeks old. Rats were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. with production license number: SCXK (Lu) 20140007. All animals were quarantined as required prior to testing. Environmental conditions for laboratory animal feeding and management:

room temperature 20 ~ 26 °C, daily temperature difference is no more than 4°C, relative humidity 40 ~ 70%, light and dark alternation time 12/12h. Animals were housed in standard rat cages, 4 rats per cage. During the period of quarantine and experiment, rats ate foods and drank water freely. The feed was for the growth and breed of SPF-level rats as well as mice and was produced by Beijing Keao Xieli Feed Co., Ltd. Drinking water was urban tap water which has been disinfected by high temperature.

Experimental grouping

SD rats were randomly divided into normal group, model group probiotic group and Wumei Pill group by random number table. Each group had 8 rats. After the period of quarantine, the other three groups except the normal group were established to be IBS-D models. All experiments were performed in accordance with the guidelines for animal research established by the Local Ethics Committee of Animal Experiments at Binzhou Medical University.

Rat IBS-D model preparation

The method of using acetic acid enema plus restraint stress is adopted in modeling (An *et al*., 2009). 24 h before the experiment, rats were prohibited for eating, but not for drinking. After ether anesthesia, the silicone tube which connected the syringe was inserted through the anus (8cm into the anus). Then, 1mL of 40mL/L acetic acid was poured into the colon, and then the silicone tube was slowly pulled out. After that, the anal was oppressed by hands and the tail was raised up for 30s. And then, the colon was rinsed with 0.01mol/L PBS1mL.When all steps were finished, the rats were put back into the cages where they could eat and drink water freely. On the 7th day, restraint stress was exerted.Rats were placed in a special transparent cylindrical drum which can restrict their limbs and trunks but not breathing. 3h later, rats were taken back to the cages. The rat fecal moisture content, rat fecal trait score, and abdominal wall withdrawal reflex (AWR) score were used to determine whether the model was successful or not (An *et al*., 2009; Zheng, 2015) .

Dosage regimen

The normal group and the model group were given normal saline, probiotic group was given probiotics, and the Wumei Pill group was given Wumei Pills. The dosage was calculated according to the weights with the formula of: dB = dA × KB / KA (dB is the daily dose per kilogram of the rat, in units of mg; dA is the daily dose per kilogram of an adult, in mg; the adult weight is calculated as 60 kg; KB = 0.71, KA = 0.11 is a constant). Continuous gavage lasted for 14d. The weight of adult is calculated as 60kg. The adult dosage of probiotics is 1 capsule/day. When this is converted to dosage of rat, it shall be 0.11 capsule/kg rat/day; as for the Wumei Pill, the adult dosage is 18g/day, and the adult weight is calculated as 60kg.Converted into rats, the dosage shall be 1936.4 mg/kg rat/day.

Detection indicators and methods

Overall conditions

Before and after modeling and treatment,observe the coat color,movement,weight and eating of all groups of rats and test Wumei Pill's effects on IBS-D rats' overall conditions.

Feces moisture content

6h defecation points: put a piece of clean filter paper in the cage to observe the feces droppings of rats within 6h; 6h loose stool rate: points sticky on paper / defecation points×100%; 24h Feces moisture content: Weigh the wet weight and dry weight of feces in each group of rats (weigh the wet weight of the feces first, and put the feces in the drying box for more than 2 h, and then weigh as well as record. This is the dry weight).The feces moisture content = (wet weight - dry weight) / wet weight × 100%.

Stool trait scores

Model rats were scored according to the Bristol Stool Scaling (Table I) and the scores were recorded. The rat fecal traits were measured at 6-8 am in the morning. At this time, the rats had more fecal matter and this was easy to collect and observe. Two people collaborated. One seized the rat and fixed its head and limbs vertically and another used the index finger and the middle finger to massage on the abdomen of the rat. And then, the rat feces was collected on a piece of filter paper for observation.

Table I.- Bristol stool scaling and scoring.

Scale	Stool trait	Score
Scale-1	Scattered dry balls, like nuts, are difficult to drain	1 score
Scale-2	Sausage-like, many pieces	2 scores
Scale-3	Sausage-like, cracked surface	3 scores
Scale-4	Sausage-like or snake-like, smooth, soft	4 scores
Scale-5	Soft blobs with clear edges (easily drained)	5 scores
Scale-6	Soft sheet, frizzy, or mushy	6 scores
Scale-7	Liquid stool samples, no fixed ingredients	7 scores

Abdominal wall retraction reflex (AWR) score

The rats were prohibited for eating but not for drinking 18 h before testing. After chloral hydrate anesthesia, the 8F catheter with a paraffin-oil coated balloon was inserted

through the anus (the end of balloon was 1cm away from the anus, and fixed at the end of the rat's tail 1cm outside the anus). Placed the rat in a self-made transparent plastic barrel cage (20cm × 8cm × 8cm), to limit it to move back and forth merely, expect turning around. The test was started 30min after the rat became adapted to the environment. Recorded the minimum amount of water that caused rat AWR, namely the minimum capacity threshold value. Each value was repeated for three times, respectively 1.0, 1.5, and 2.0 mL, and each of them lasted for 30 s with an interval of 5 min. The obtained data were averaged. The standard of AWR score: 0 points: the mood of the rats is basically stable when the colorectal distension is stimulated; 1 point, rats' mood became unstable when given stimulation, and occasionally twisted the head; 2 points, muscles of the back and abdomen contracted slightly but the abdomen did not raise above ground; 3 points, muscles of the abdomen and back strongly contracted and lifted the abdomen off the ground; 4 points, abdominal muscles contracted strongly, the abdomen bowed and the abdomen as well perineal region were lifted off the ground.

Determination of serum level of TNF-α, IL-6 and LPS

24 h after the final dose, rats were injected 10% chloralic hydrate subcutaneously for anaesthesia. Drawed blood from abdominal aortics and their centrifugal serum was reserved. Contents of rats' serum level of TNF-α, IL-6 and LPS were determined by using ELISA kits.

Determination of colonic mucosal tissue TLR4/9 and NF-kB

Collect the colonic tissue specimen and determine contents of colonic mucosal tissue TLR4/9 and NF-kB of each group of rats through the immunohistochemistry and Western blot after the treatment.

For changes of the colonic mucosa, Collect the colonic tissue specimen and then fix, dehydrate and slice it. Observe the colonic tissue morphology under the microscope if it has the edema, hyperemia, inflammatory cell infiltration and other pathologic changes so as to exclude organic pathologic changes.

Statistical methods

SPSS 19.0 statistical software was used for data analysis. The measurement data were analyzed using the t-test of the sample mean. Comparisons between groups were analyzed by analysis of variance. The results were expressed as mean ± standard deviation (±s). When $P<0.05$, it was deemed as that the difference was statistically significant.

RESULTS

General conditions of rats

No rats died during the experiment. Normal group: The rats had good mental status, the normal capacity for eating and movement, and a sensitive response. The fecal traits were normally granular and the fur was neat and shiny. Model group: After 3 days from the start of the modeling, some rats began to suffer from mental retardation, wilting, reduced movement and capacity for eating, arched backs, unsmoothed fur and less luster, diarrhea, loose stools, and loose stools near the anus. No recovery was found at the end of the trial. In the probiotic group, the symptoms were the same as those in the model group during the modeling period. From the 3rd day after intragastric administration, the diarrhea of some rats gradually decreased, the mental state of the rats increased, the activity gradually increased, and the coat was much tidy but less glossy. At the end of the experiment, there was still a small part of the rat's feces traits. In the Wumei Pill group, symptoms were the same as those in the model group during the modeling period. Rats' diarrhea gradually relieved the third day after gavage, and the fecal traits changed from unshaped to half-shaped and shaped finally. The mental state of rats gradually increased and the movement gradually increased. At the end of the experiment, there was still a small part of rat's feces trait was slightly watery.

Weight changes of rats

Compared with normal group, the weights of the model group of rats, probiotic group and the Wumei group of rats decreased clearly after modeling, and there was the significant difference ($P<0.05$).Compared with the model group during dosing, the weights of probiotic group and the Wumei group of rats increased to different extent, and the weights had the significant difference between dosing for 7 days and for 14 days ($P<0.05$) (Table II).

Table II.- Weight changes of all groups of rats.

Group	1 day (g)	7 days (g)	14 days (g)
Normal group	75.59±0.59	89.96±0.62	101.76±1.04
Model group	68.92±0.46*	76.11±0.52*	85.36±0.80*
Probiotics group	69.05±0.52*	80.38±0.64*$^{\Delta}$	91.27±1.11*$^{\Delta}$
Wumei Pill group	69.09±0.51*	83.50±0.49*$^{\Delta}$	95.39±0.75*$^{\Delta}$

Compared with normal group,*$P<0.05$; compared with model group, $^{\Delta}P<0.05$.

Rat fecal trait scores

Rat fecal trait scores were essentially the same in

all groups before modeling, and there was no statistical difference. After modeling, compared with the normal group, the fecal trait scores of the model group, the probiotic group, and the Wumei Pill group were all significantly higher (P<0.05); after the completion of the administration, Compared with the model group, the fecal trait score of each group decreased to varying degrees with significant differences (P<0.05), indicating that the probiotics and Wumei Pills could all alleviate the diarrhea symptoms of the model rats (Table IV).

Table III.- Feces moisture content changes of all groups of rats.

Group	After modeling			After administration		
	6h defecation points	6h stool rate	Feces moisture rate	6h defecation points	6h stool rate	Feces moisture rate
Normal group	5.75±1.58	2.08±5.89	29.92±2.69	5.13±1.36	1.56±4.42	31.31±4.00
Model group	12.75±2.71*	54.03±9.49*	63.05±6.05*	13.13±2.53*	54.81±8.94*	62.80±7.53*
Probiotics group	13.38±2.62*	53.03±10.53*	64.98±6.72*	10.00±1.41$^{\Delta}$	38.02±6.18$^{\Delta}$	48.45±7.09$^{\Delta}$
Wumei Pill group	13.63±2.88*	57.06±7.05*	65.02±5.72*	9.75±1.91$^{\Delta}$	30.66±11.00$^{\Delta}$	47.23±6.54$^{\Delta}$

Compared with normal group, *P<0.05; compared with model group, $^{\Delta}$P<0. 05.

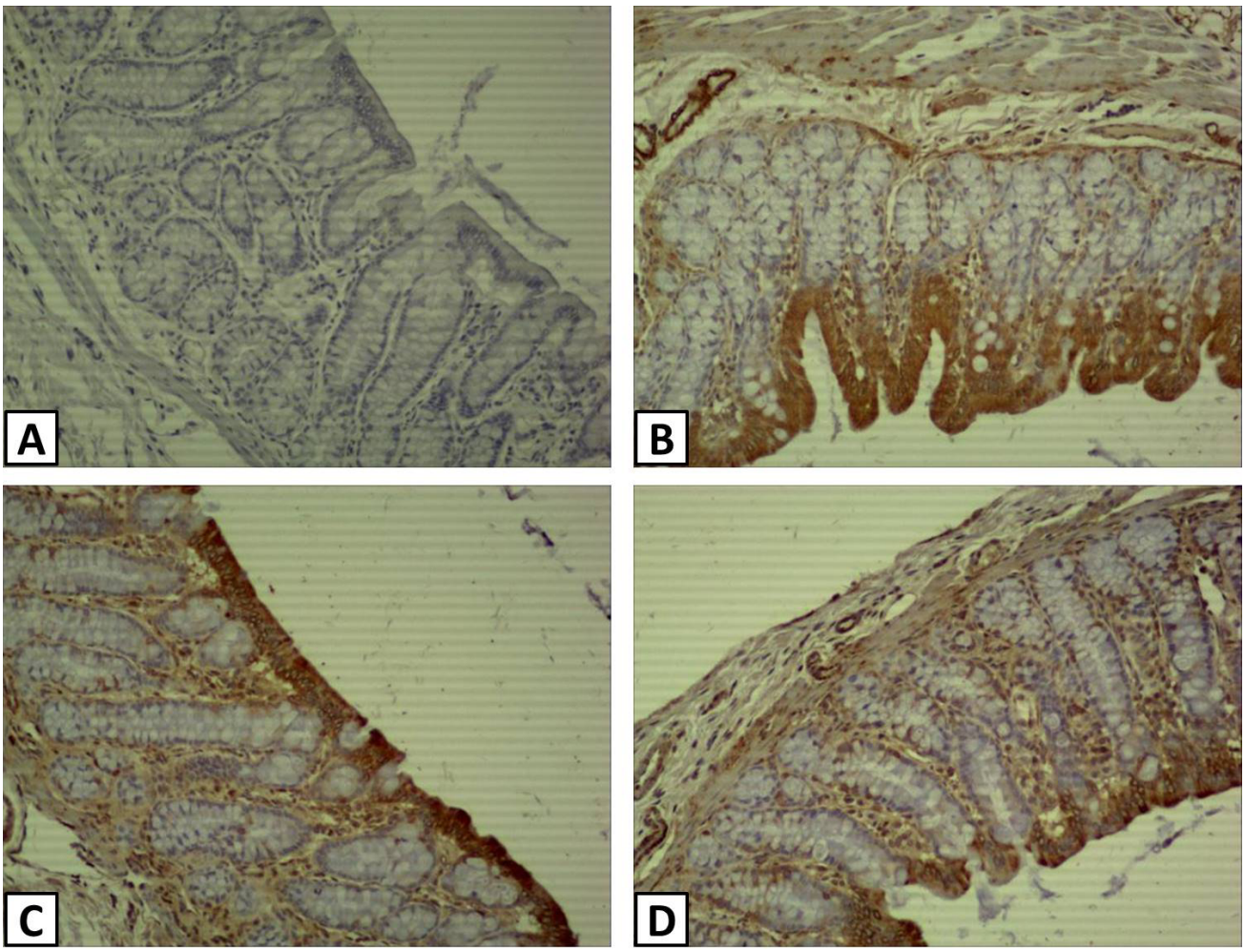

Fig. 1. Effect of Wumei Pill on TLR4 expression in chronic mucosal tissue of normal group (A), model group (B), probiotics group (C) and Wumei Pill group (D) rats with dirrhea-predominant irritable bowel syndrome. Magnification: all 100X.

Table IV.- Fecal trait scores of all groups of rats.

Group	Before Modeling	After modeling	After administration
Normal group	3.25±0.71	3.63±0.92	3.38±0.74
Model group	3.38±0.52	6.13±0.83*	6.38±0.74*
Probiotics group	3.00±0.76	6.38±0.74*	5.25±1.04$^{\Delta}$
Wumei Pill group	3.38±0.52	6.50±0.76*	5.00±0.76$^{\Delta}$

Compared with normal group,*P<0.05; compared with model group, $^{\Delta}$P<0. 05.

AWR scores

After modeling, compared with normal group, the AWR scores of the model group, probiotic group, and Wumei Pill group were significantly higher than that of the normal group (P<0.05). After the completion of administration, compared with the model group, the AWR scores of each volume decreased to some extent, and some of them had significant differences (P<0.05), indicating that probiotics and Wumei Pills were able to reduce the organ sensitivity of model rats (Table V).

Contents of rats' serum level of TNF-α, IL-6 and LPS

Compared with the normal group, contents of rats' serum level of TNF-α, IL-6 and LPS of the model group were significantly higher than that of the normal group, and the difference had the statistical significance (P<0.05). After dosing, comparing with the model group, contents of TNF-α,IL-6 and LPS of probiotics group and Wumei pill group decreased to different extent, and the Wumei pill group had significant differences (P<0.05),indicating that Wumei Pills were able to reduce contents of rats' serum level of TNF-α,IL-6 and LPS (Table VI).

Colonic mucosal tissue TLR4/9 and NF-kB

Colonic mucosal tissue TLR4/9 and NF-kB were determined through immunohisto chemistry and observed under the microscope. The normal group did not show a positive reaction while the model group, probiotics group and Wumei Pill group tested positive. Besides, the positive reaction of model group was the most serious, and the positive reaction's severity of probiotics group and Wumei Pill decreased (Figs. 1, 2, 3).

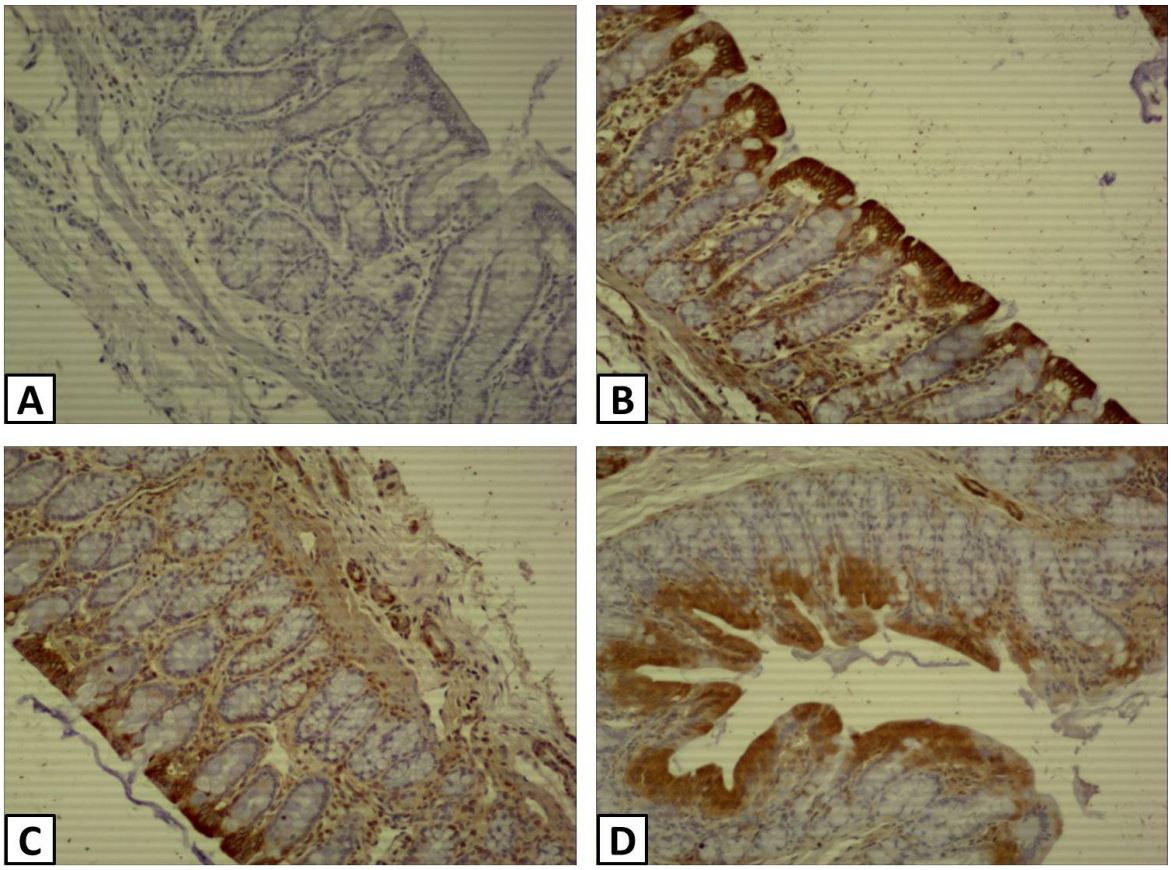

Fig. 2. Effect of Wumei Pill on TLR9 expression in chronic mucosal tissue of normal group (A), model group (B), probiotics group (C) and Wumei Pill group (D) rats with dirrhea-predominant irritable bowel syndrome. Magnification: all 100X.

Table V.- AWR scores of all groups of rats.

Group	After modeling			After administration		
	1ml	1.5ml	2ml	1ml	1.5ml	2ml
Normal group	0.58±0.24	1.25±0.39	2.17±0.31	0.63±0.33	1.33±0.50	2.33±0.36
Model group	1.13±0.25*	2.04±0.21*	3.08±0.35*	1.42±0.39*	2.17±0.25*	3.25±0.30*
Probiotics group	1.17±0.44*	2.21±0.47*	3.13±0.43*	0.96±0.52	1.79±0.50	2.63±0.38$^{\Delta}$
Wumei Pill group	1.13±0.35*	2.13±0.35*	3.08±0.24*	1.00±0.36$^{\Delta}$	1.79±0.31$^{\Delta}$	2.75±0.30$^{\Delta}$

compared with normal group, *P<0.05; compared with model group, $^{\Delta}$P<0. 05.

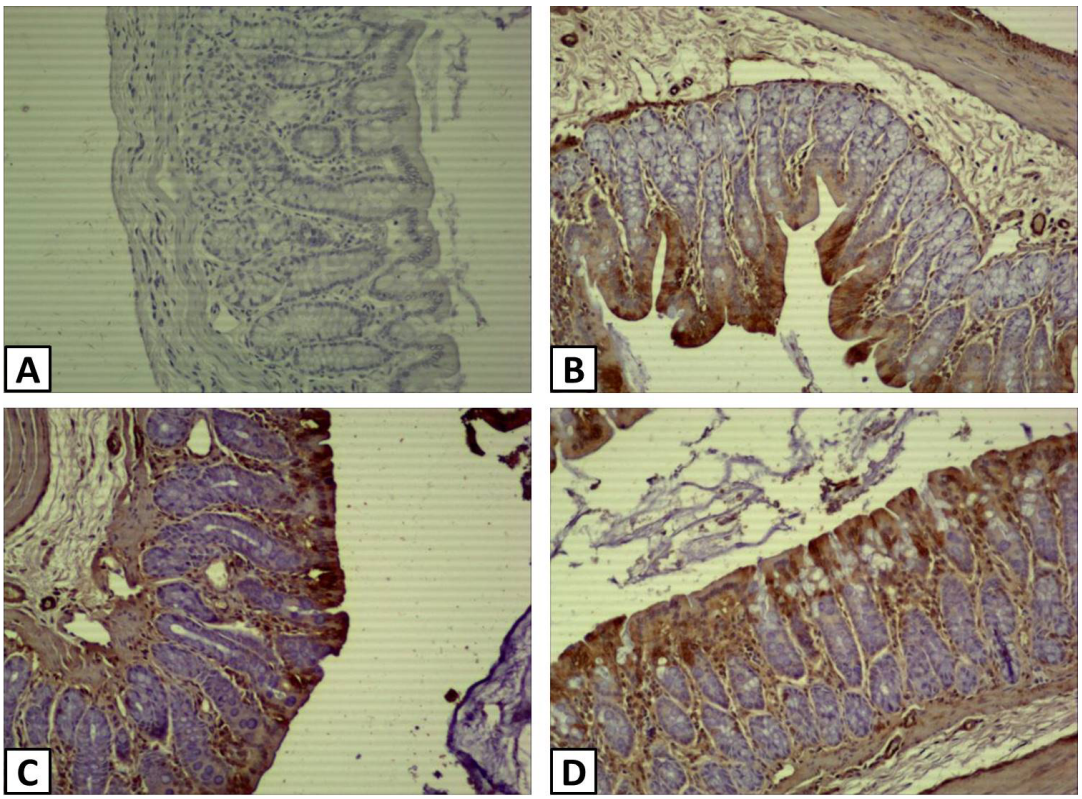

Fig. 3. Effect of Wumei Pill on NF-kB expression in chronic mucosal tissue of normal group (A), model group (B), probiotics group (C) and Wumei Pill group (D) rats with dirrhea-predominant irritable bowel syndrome. Magnification: all 100X.

Table VI.- Contents of serum TNF-α, IL-6 and LPS of all groups of rats (ng/L).

Group	TNF-α	IL-6	LPS
Normal	449.03±96.50	232.48±34.72	609.86±84.80
Model	666.50±109.59*	345.81±60.17*	791.81±80.77*
Probiotics	597.36±99.21*	311.20±46.73*	713.67±61.30*
Wumei Pill	538.05±120.99$^{\Delta}$	281.96±58.79$^{\Delta}$	675.67±61.01$^{\Delta}$

Compared with normal group,*P<0.05; compared with model group, $^{\Delta}$P<0. 05.

The expression of TLR4/9 and NF-kB protein in all groups of rats' colonic tissues was determined by western blot method, and the result was showed in Figure 4. The grey value determination and quantitative analysis of the protein electrophoresis band was made through the software Scion Image (Table VII).

From Table VII, compared with the normal group, the content of the colonic tissue TLR4/9 of the model group rats determined by western blot increased obviously, and the difference was statistically significant (P<0.05). After dosing, comparing with the model group, contents of TLR4/9 of probiotics group and Wumei Pill group decreased to different extent, and the difference was also significant (P<0.05). Compared with the normal group, the content of colonic tissue NF-kB of the model group of rats increased obviously, and the difference was statistically significant (P<0.05).Compared with the model group after

dosing, the content of NF-kB of the Probiotics group and Wumei Pill group decreased and there was a significant difference in Wumei Pill group (P<0.05),indicating that Wumei Pills had the significant effects on reducing contents of rats' colonic tissue TLR4/9 and NF-kB (P<0.05).

Table VII.- Grey values of TLR4/9, NF-kB protein expression tested by western blot.

Group	TLR4	TLR9	NF-kB
Normal group	0.83±0.17	1.92±0.39	0.51±0.14
Model group	2.58±0.15*	5.11±0.38*	0.99±0.20*
Probiotics group	1.24±0.12*$^{\Delta}$	2.88±0.13*$^{\Delta}$	0.81±0.15*
Wumei Pill group	1.02±0.18$^{\Delta}$	2.18±0.16$^{\Delta}$	0.65±0.12$^{\Delta}$

Compared with normal group, *P<0.05; compared with model group, $^{\Delta}$P<0. 05.

Changes of colonic mucosa

Collect colonic tissues of all groups of rats and observe them by eyes. Compared with the normal group, the colonic tissue of the model group of rats had the hyperemia and edema obviously, while the degree of hyperemia and edema of the probiotics group and Wumei Pill group weakened than that of the model group (Fig. 5).

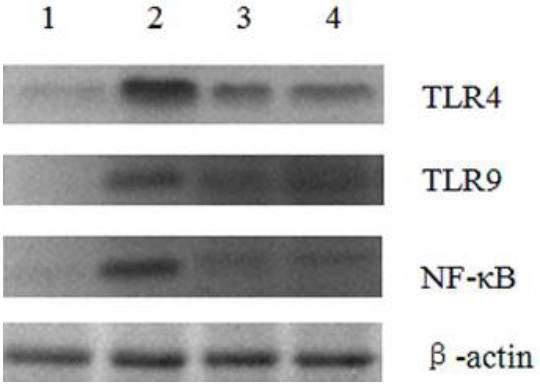

Fig. 4. Wester Blot showing expression of TLR4, TLR9 and NF-kB in colonic tissue of normal (1), Model (2), Probiotics (3) and Wumei Pill (4) groups of rats with dirrhea-predominant irritable bowel syndrome.

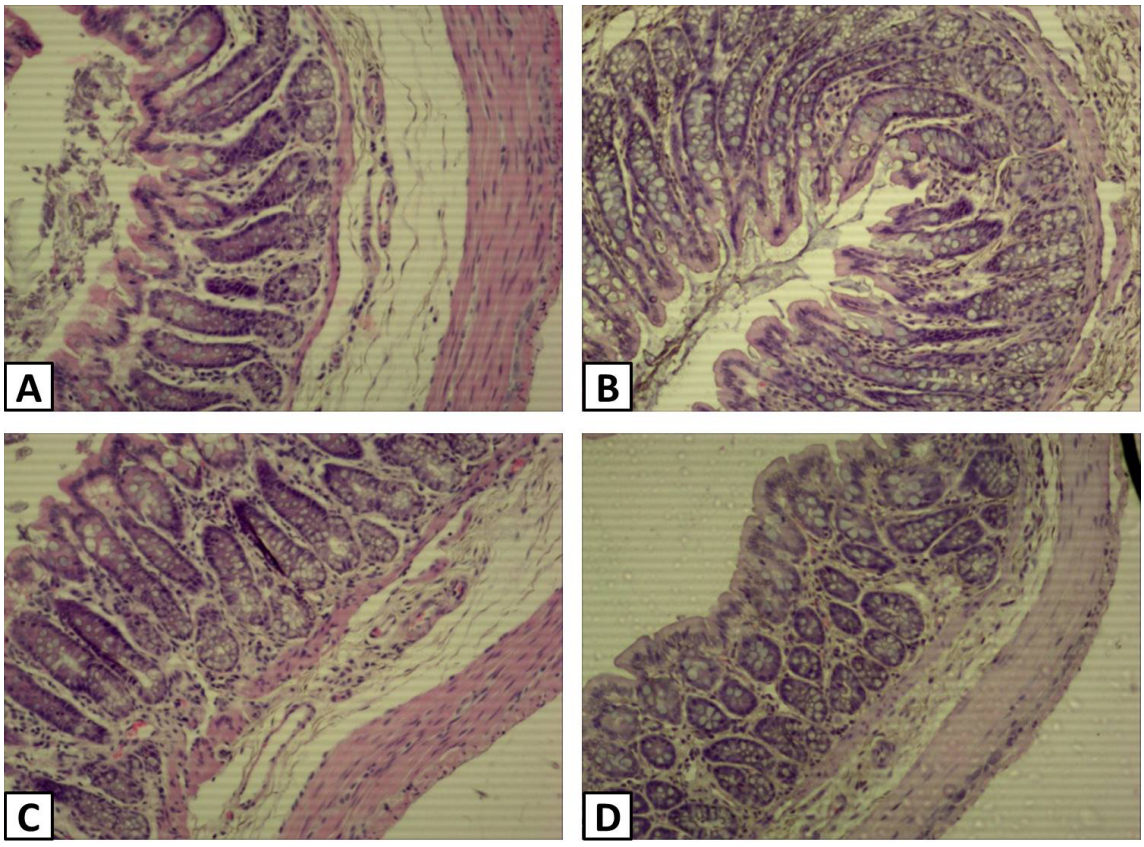

Fig. 5. Pathological changes in colonic tissue of normal group (A), model group (B), probiotics group (C) and Wumei Pill group (D) rats with dirrhea-predominant irritable bowel syndrome.

Fix, dehydrate and slice colonic tissues of all groups of rats and then observe them under the microscope. The structure of the colonic tissue of the normal group's rats was normal, and this tissue had the complete 4-layer structure, including the mucosal layer, mucosal lower-layer, substratum, and serosal layer. It could be seen in the model group that the serosa became thick, and the convective tissue became loose and oedematous. Besides, the lymphocytes infiltration appeared. The infiltration of inflammatory cells appeared in the probiotics group and Wumei Pill group (Fig. 5).

DISCUSSION

IBS-D belongs to common diseases in digestive system, and its morbidity goes up year after year. The pathogenesis of IBS-D has not completely clarified. Currently this disease is considered to be related to the intolerance of diet, abnormalities of gastronintestinal motility, changes of internal organs' susceptibility, inflammation, intestinal infection, abnormalities of brain-gut axis, mental and psychological disorders, genetic susceptibility, intestinal flora imbalance and other factors (Tanaka *et al*., 2011; Ge and Xu, 2013; Zhang and Song, 2014). TNF-a, IL-6 and other inflammatory cytokines inside IBS-D patients' bodies always increase to different extent (Liebregts *et al*., 2007). TLRs have the close relation with inflammatory diseases clinically.as the receptors of feeling bacterial toxins, the family of Toll-like receptors can distinguish bacterial LSP molecules in pathogenic microbes, so that play the crucial role in the inflammatory signal control (Oscar *et al*., 2010). LPS-TLR4/NF-kB has the close relation with the anti-inflammatory immunization (He and Huang, 2013). Li *et al*. (2014) find that the activation of TLR4 signaling pathway exists in IBS-D patients' bodies and rats' colonic mucosal tissues and TLR may have significant effects on the occurrence and development of IBS by mediating the muscosal immune, through the inclusion of IBS-D patients and modeling IBS-D rats. IBS-D belongs to the category of "diarrhea" in Traditional Chinese medicine. Wumei Pills was mentioned in No.338 article of Zhang Zhongjing's Treatise on Exogenous Febrile Disease. The article states that Wumei Pills "Zhu Jiu Li (meaning: mainly for treating the chronic diarrhea)". "Jiu Li" is also called the chronic diarrhea. And the Wumei Pill is an effective prescription for the treatment of chronic diarrhea. Wumei Pill consists of 10 medicinal herbs including ebony, asarum, dried ginger, berberine, cork, angelica, aconite, peony, cassia twig, and ginseng. At present, experimental studies (Ding and Dong, 2017) have shown that Wumei Pill has functions of anti-inflammatory, immune regulation, promote gastrointestinal function recovery, regulate intestinal flora, inhibit apoptosis, repair mucosal barrier and anti-oxidative damage.

CONCLUSIONS

This study has shown that the Wumei Pill Rat can decrease the Fece moisture content, fecal trait scores and AWR scores ($P<0.05$), and has the function of alleviate the syndrome of diarrhea. Besides, the mechanism of action of the Wumei Pill is possibly related to restraining the transmission of TLR4/9-NF-kB signaling pathway and reducing the release of pro-inflammatory cytokines and bacterial endotoxins.

ACKNOWLEDGEMENTS

We acknowledge the financial supports of the National Natural Science Foundation of China under Grant No. 81703983, Project of scientific and technological development of traditional Chinese medicine in Shandong under Grant No. 2017-232. Financial support for youth backbone teacher training program of Binzhou Medical University in 2017.

Statement of conflict of interest

We declare that there is no conflict of interests regarding the publication of the manuscript.

REFERENCES

An, Y., Bai, D.Q., Fu, J. and Sun, X.N., 2009. Establishment and evaluation of irritable bowel syndrome model induced by acetic acid enema plus restraint stress in rats. *World Chinese J. Digestol.*, **17**: 1548-1551. https://doi.org/10.11569/wcjd.v17.i15.1548

Ding, X.J. and Dong, Z.P., 2017. Experimental research progress of Wumei Pills. *China Med. Herald*, **14**: 52-55.

Ge, J.B. and Xu, Y.J., 2013. *Inernal medicine,* 8th Edition. People's Medical Publishing House, China.

He, J.X. and Huang, L.H., 2013. Research development of LPS-TLR4/NF-kB signaling pathway and anti-inflammatory immunization. *Chinese Foreign Hlth. Abst.*, **37**: 27-29.

Li, B., Sun, N. and Gu, S., 2016.Wumei Pill and its divided formula's effects on inflammatory factors of rat's ulcerative colitis and TLR9/MyD88/NF-kBp65 signaling pathway. *China J. Trad. Chinese Med. Pharm*., **31**: 1901-1905.

Liebregts, T., Adam, B., Bredack, C., Roth, A., Heinzel, S., Lester, S., Downie-Doyle, S., Smith,

E., Drew, P., Talley, N.J. and Holtmann, G., 2007. Immune activation in patients with irritable bowel syndrome. *Gastroenterology*, **132**: 913-920. https://doi.org/10.1053/j.gastro.2007.01.046

Li, M., Lv, B. and Chu, L., 2014. Aviation of toll-like receptor 4 signaling pathway was existed in IBS-D. In: *Seventh Academic Meeting on Digestive System Diseases in Zhejiang Province*. First American Gastroenterological Association Academic Forum in China, Zhejiang, China.

Luo, J.H., Xiao, B. and Zheng C.X., 2016. Treatment of irritable bowel syndrome of cold and heat misdiagnosis diarrhea with Wumei Pill and Chinese herbal application. *Hubei J. Trad. Chinese Med*., **38**: 32-34.

Ni, S.W. and Sun J.C., 2014. Clinical study on Wumei Pills treatment of irritable bowel syndrome with cold, hot, miscellaneous and diarrhea type of irritable bowel syndrome. *Trad. Chinese Med. Bull. China*, **13**: 53-58.

Oscar, R.F., Joselin, H.R. and Max, S., 2010. From Cytokines to Toll-Like receptors and beyond-current knowledge and future research needs in irritable bowel syndrome. *J. Neurogastroenterol. Motil*., **4**: 363-373.

Tanaka, Y., Kanazawa, M., Fukudo, S. and Drossman, D.A., 2011. Biopsychosocial model of irritable bowel syndrome. *J. Neurogastroenterol. Motil*., **17**: 131-139. https://doi.org/10.5056/jnm.2011.17.2.131

Yan, S.G., 2012. *Experimental research of Wumei Pill and its divided formula's effects on cell factors of rat's ulcerative colitis and TLR4/ NF-kB signaling pathway*. PhD thesis, Chengdu University of Traditional Chinese Medicine, Chengdu, China.

Zhang, L. and Song, J., 2014. New progress in the study of irritable bowel syndrome. *Chinese J. clin. Gastroenterol*., **26**: 137-138.

Zheng, L., 2015. *Effect of Tongxie Yaofang prescription on serum 5-HT and VIP levels in rats with diarrhea-predominant irritable bowel syndrome*. PhD thesis, Chengdu University of Chinese Medicine, Chengdu, China.