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Protective Effects of *Atractylodes macrocephala* Polysaccharide on iIELs and the *IL-6*, *TNF-α* mRNA Level in *E. coli* induced Diarrheal Mice

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Authors' Contribution
JL conceived and designed the research. QZW and WJZ performed the experiment. QZW analyzed the data and samples. QZW and RLY wrote the manuscript. FN, SA and YFS critically revised the manuscript. All authors approved the final version.

Key words
Atractylodes macrocephala polysaccharide, Lymphocytes, IL-6, TNF-α

ABSTRACT

Study was carried out to investigate the protective effects of Atractylodes macrocephala polysaccharide (AMP) on the quantity of intestinal intraepithelial lymphocytes (iIELs), and the *IL-6*, *TNF-α* mRNA transcription level in duodenum of diarrheal mice. A total 72 mice were randomly divided into 6 groups: control (CG), infection (IG), positive drug (PG), AMP low/middle/high dose (LG/ MG/ HG) groups respectively, (n=12). The mice were induced by diarrhea with *E. coli* (5×10^7CFU) via i.p injection (0.5 mL/time/d) after seven days of pre-administration except in CG. After the induction of the diarrhea, the pathological signs and the quantity of iIELs in small intestine and the mRNA level of IL-6 and TNF-α in duodenum were analyzed by qRT-PCR from all groups at 6 h and 4 d, respectively. IG group shows abnormal mental state and the lack of the appetite; however, AMP pre-treatment caused the normal state and appetite. The duodenal mucosa in IG was seriously injured; with epithelial hyperplasia and necrosis. However, the intestinal histology of duodenal mucosa was reverted by AMP pre-treatment with normal structure and morphology with protective rate of 0 %, 70 %, 66.7 %, 40 % and 33.3 %, from IG, PG, HG, MG and LG groups respectively at 6 h. As compared to CG, the quantity of iIELs in IG was significantly (P<0.01) higher at 6 h and 4 d in duodenum and ileum of diarrheal mice; however, the iIELs quantity in IG was significantly (P<0.01) higher and lower at 6 h and 4 d in jejunum, respectively. The AMP pre-treatment reduced iIELs in duodenum and ileum at 6 h and 4 d, respectively and increased iIELs in jejunum at 6 h and 4 d. The mRNA level of *IL-6* and *TNF-α* in duodenum was increased in IG at 6 h and 4 d as compared to CG (P<0.01). Although the *IL-6* and *TNF-α* in levels in duodenum decreased significantly with AMP pre-treatment as compared with IG (P<0.05). Results suggested AMP showed anti-diarrheal activity which maintained immune barrier in intestinal mucosa through regulating the quantity of iIELs and soothing the duodenal inflammatory injury might via NF-κB inflammatory pathway.

INTRODUCTION

Diarrhea is a common problem with public health and animals, one of the leading causes of antibiotic-associated and widespread around the world (Alebel *et al*., 2018; Chang *et al*., 2018) results in high morbidity and mortality. Many pathogens and several emerging species threat the animal production and losses the livestock mainly in developing countries (Dunn and Johnson, 2018). Enterotoxigenic *Escherichia coli* is well-known pathogen that cause diarrhea. Specifically, it adheres to the intestinal mucosa, then secretes enterotoxin and adhesins, conclusively changes the intestinal permeability and disturbs water and electrolytes balance (Mirhoseini *et al*., 2018).

The gut is frequently exposed to food, symbiotic microorganisms, and environmental agents (Kao, 2013) and also serves as a main channel of entry for *E. coli*. A crucial defense mechanism against *E. coli* invasion in the gut is the single layer of epithelial cells that separates the intestine lumen from the hypo of tissues (Peterson and Artis, 2014). The barrier function of the intestinal epithelium is supported by cells and soluble factors of the

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intestinal immune system (Kamada *et al*., 2013). Chief among them are intestinal intraepithelial lymphocytes (iIELs), which are implanted in the intestinal epithelium and represent one of the single largest populations of lymphocytes in the body (Van Kaer and Olivares-Villagómez, 2018). iIELs could be divided into type a or conventional T cells and type b or nonconventional T cells based on T-cell receptor (TCR) usage and expression of coreceptors (Taveirne *et al*., 2011). CD4+T cells in iIELs could attenuate the adjusting of inflammation and immunity, which enhanced the effect of self-protection (Cheroutre, 2005). Furthermore, report indicated that the diarrhea induced by LPS (the main structure of *E. coli*) could be cured by plants polysaccharides as well (Sousa *et al*., 2016). LPS-induced tissue damage related to NF-κB inflammatory signaling pathway which prevailingly mediated by TNF receptor associative factors, such as IL-6, IL-8 and TNF-α.

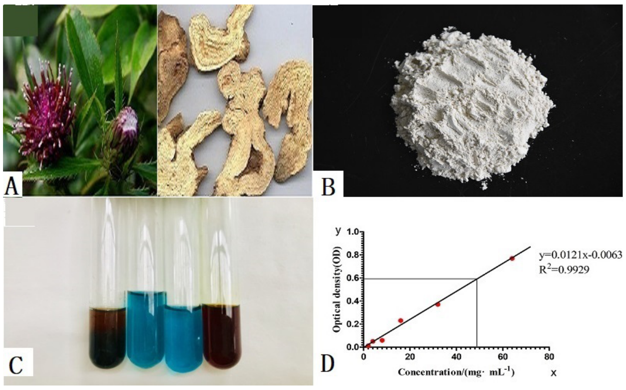

Fig. 1. Identification of AMP.
A, the plant and administration parts of *Atractylodes macrocephala*. B, the entity of AMP. C, the chemical identification of AMP (1 Molish test (+), 2 biuret reaction (-), 3 Fehling's reagent reaction (-), 4 iodine-potassium iodide reaction (-). Note: "+" positive, "-" negative). D, the standard curve of glucose.

A. macrocephala (Fig. 1A) is a traditional Chinese medicine that derived from the rhizoma of *A.macrocephala* Koidz., used to treat gastrointestinal ulcers, lower immunity, chronic wasting disease, cancers and diarrhea (Guan *et al*., 2018; Huang *et al*., 2005; Zhang *et al*., 2017). *A. macrocephala* mainly contains polysaccharide, lactone, volatile oil and sesquiterpenes (Chou *et al*., 2012; Kang *et al*., 2011), which is used as an anti-bacteria, anti-inflammation and anti-tumor properties (Dong *et al*., 2008; Zhang *et al*., 2000). In our previous research, we confirmed that the extract of zhuqin compounds enhanced the immunity of immunosuppressed in mice (Juan *et al*., 2015). Polysaccharides as the vital material basis for Chinese herbal medicine perform unique therapeutic effects such as regulating immunity, apoptosis and anti-tumor mediator (Wang *et al*., 2018; Wei *et al*., 2018; Li *et al*., 2018; Fan *et al*., 2017; Xu *et al*., 2014). Previously we found that the total polysaccharides of *Scutellaria* and *A. macrocephala* (including AMP) maintained the integrity of immune function via inhibiting the autophagy of spleen lymphocytes in mice (Pang-min, 2018). Therefore, the aim of this study was to investigated the effects of AMP on *E. coli* induced diarrhea on the ability to affect the quantity of iIELs in small intestine and the mRNA transcription of *TNF-α* and *IL-6* in duodenum of mice.

MATERIALS AND METHODS

Drugs and kits

A. macrocephala was purchased from Sichuan Qianjin Zhongyao Co., Ltd. (batch No.: 150601). Astragalus *polysaccharide* oral liquid, purchased from Henan Anpu Biotechnology Co., Ltd. (batch No.: (2015) 16270523). Pathogenic *E. coli* O_{101} strains (CVCC231), was purchased from the China Veterinary Medicine Supervision Institute for strain preservation. B511321-UNIQ-10 total RNA extraction kit was purchased from Sangon Biotech Co., Ltd., Shanghai, China. Primer-Script™ reagent kit was purchased from TakaRa Biotechnology Co. Ltd., Beijing, China. Quantitative PCR kit was purchased from TransGen Biotechnology Co. Ltd., Beijing, China.

Preparation and identification of AMP

AMP was prepared according to the previous study (Pang-min, 2018). AMP was primitively identified by the tests of molish, biuret reaction, Fehling reagent reaction and iodine-potassium iodide reaction. Phenol-sulphate acid method (Zhao, 2005) was adapted to measure the purity of AMP. The average absorbance (AA) of 2, 4, 8, 16, 32, 64 mg/ml glucose solution were collected, and then the standard curve was drawn. The AA of AMP was measured and the polysaccharides concentration was calculated by the standard curve, and the purity (m%) followed the formula: $m\% = 100 \times m_1/m_2$ [m_1: polysaccharides concentration; m_2: AMP concentration (50.4 mg/L)].

The structure of AMP analysis by fourier transform infrared spectrometry (FTIR)

A small amount of AMP (3 mg) were weighed and added to KBr, then crushed into thin slices. FTIR technique was used to scan infrared wave number in the range of 4 000 cm^{-1}-400 cm^{-1}, and the height ratio of characteristic peak was calculated and analyzed by FTIR.

Animals and treatment

A total 72 Kunming strain mice (20±2 g, male and female in equal), were purchased from the experimental animal, Center College of Animal Science, Southwest University Chongqing China. After one-week adaptive feeding, mice were randomly divided into 6 groups equally (n=12). The positive drug group (PG) was administered with 1.2 mg/mL astragalus polysaccharide orally, while the AMP low, medium and high dose groups (LG, MG, HG) were administered with 1.2, 3.6, and 6 mg/mL of AMP, respectively. The control group (CG) and infection group (IG) were administered with equal volume of normal saline. Mice were administered with *E. coli* 0.5 mL/time/day for 7 days. All experiments were. All animal research experiments were conducted according to the National Institutes of Health guidelines for experimental animal use and were approved by the Institutional Animal Care and Use Committee at the Southwest University, Rongchang, Chongqing China. Mice were housed at standard conditions (23±2 °C; 60% humidity; 12 h light-dark cycle) and fed *ad libitum* standard diet during the whole experiment period. After the last administration subsequent 24 h, mice in each group were intraperitoneally (i.p) injected with *E. coli* (5×10^7 CFU) to induce diarrhea except CG.

Clinical observation and management

During the clinical observations when mice show the depressed spirit, decreased appetite and laxly in IG the point time was set as 0 h. The incidence of diarrhea was calculated at 6 h. To evaluated the protective effect of the drugs on diarrheal mice, we set fecal consistency of scores in mice according to the previous method (0, normal; 1, soft faeces; 2, mild diarrhea; and 3, severe diarrhea) (Liu *et al*., 2016). The fecal consistency of scores in mice were collected at 4 d by a trained individual, but who had no prior knowledge of treatment each mouse had received.

The quantity of iIELs in small intestine analyzed by moditec

At 6 h and 4 d, 6 mice from each group were euthanized by cervical dislocation. Intestinal segment (duodenum, jejunum, and ileum) samples were fixed in 10% Formalin solution, embedded paraffin, sectioned, stained with H and E staining (Estrada *et al*., 2005). The quantity of iIELs between 100 columnar epithelial cells in intestinal mucosa was counted by Moditec photo processing software under the light microscope (Jin-kun *et al*., 2008). Three distinct regions of each intestinal segment were selected as average value for iIELs analysis.

IL-6 and TNF-α mRNA expression level in duodenum

At 6 h and 4 d, the duodenal segments from 6 mice from CG, IG, PG and HG were collected. Duodenal segments were rinsed with PBS buffer to clean the intestinal contents, put into the EP tube (without RNA enzyme), and stored at -80 °C for further analysis. The primers selected for the current study was designed by GenBank, (Table I). Duodenal tissue samples (50mg) were collected for the synthesis of RNA and cDNA. RNA was extracted by B511321-UNIQ-10 total RNA extraction kit, and reverse transcribed into cDNA by primer-script™ reagent kit. qRT-PCR reaction was performed briefly a total reaction volume system (20 µL) contains 2 × SYBR premix ex Taq mix 10 µL, QF (10 µmol/L), QR (10 µmol/L) respectively 0.5 µ L, the template cDNA 2 µL, and 7 µL RNase free dH_2O. The amplification procedure parameters contain the 94 °C for 30 s, 94 °C 5 s, 61 °C 35 s, with 40 cycles, then the specificity of products were detected by dissolution curve. Dissolution parameter contains the: 97 °C for 10 s, 65 °C 60 s, 97 °C 1 s, 1 cycle. The ΔCt value of the amplification each curve of cDNA sample was recorded, and the relative level of mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method (Ghowsi *et al*., 2018).

Statistical analysis

The data were expressed by mean ± standard deviation, and analyzed by SPSS 20.0 statistical software, $P < 0.05$ was the significant difference, $P < 0.01$ was the highly significant difference.

RESULTS

Identification of AMP

As shown in Figure 1, The polysaccharides concentration (49.454 5 mg/L) was calculated, and the purity (m%) of AMP was 98.12%. The results illustrated that AMP contained polysaccharide, instead of proteins, reducing sugars and starch. As shown in Figure 2, the strong vibrations arose in characteristic spectral band of 3 446.61 cm^{-1} and 3 351.41 cm^{-1} indicated that the stretching vibration absorption peaks of -OH. The characteristic spectral band of 2 934.88 cm^{-1} and 1 637.69 cm^{-1} severally signified absorption peak of $-CH_2$ and -COOH. The -C-H vibration absorption peak at 1 402.64 cm^{-1}, suggested that AMP included ring structure with carbon atom. The spectral band of 1 131.90 cm^{-1}, 1 036.69 cm^{-1} and 941.49 cm^{-1} were the characteristic absorption peaks of pyranose. The spectral band of 816.53 cm^{-1} was absorption peak of alpha-glycosidic bond. The results implied that AMP might be a class of pyran polysaccharide with alpha-glycosidic bond.

Protective effect of AMP on diarrheal mice

During the whole period of treatment the mental state, drinking, appetite and physical activity of mice in each group was normal with no signs of diarrhea. At 6 h observation, the mental state and appetite of mice in CG was normal. Conversely, the mice showed the abnormal mental state and the lack of the appetite in IG. Although, the abnormal mental state and appetite of mice in PG, LG, MG and HG group's shows nearly to the normal levels. The incidence and protective rate of diarrhea in each group is shown in Table II. The fecal score of IG was significantly increased ($P<0.01$) as compare to CG. As compared with IG, the faecal score of PG, LG, MG and HG groups were decreased significantly by dose-dependently decreased ($P<0.01$) (Table II).

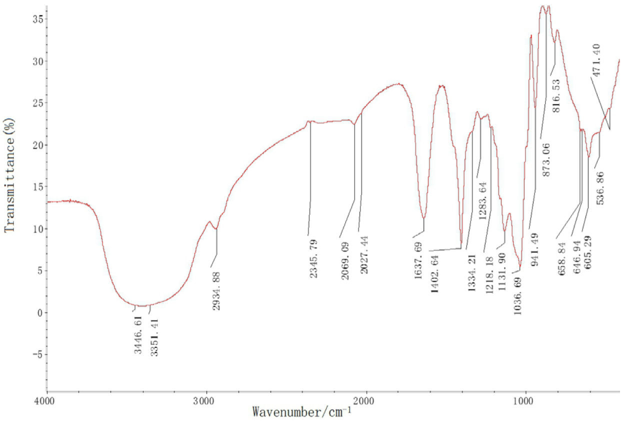

Fig. 2. Infrared analysis atlas of AMP.

Protective effect of AMP on the intestinal histology

Duodenal mucosa in CG was clear polarized, the morphology of intestinal epithelial cells was normal, the nucleus with dark blue was localized at the base of the enterocyte and the cytoplasm was seems uniformly red stained. The duodenal mucosa in IG was seriously injured; the intestinal epithelial cells show hyperplasia, flattened crypts, pyknosis and necrosis. However, PG, LG, MG and HG groups' intestinal histology were improved and duodenal mucosa with normal structure of villi and crypts, and blue stained nuclei and normal morphology (Fig. 3).

Quantity of iIELs in small intestine of E. coli *induced diarrheal mice*

At 6 h, the quantity of iIELs in small intestine of IG was significantly higher than that in CG ($P<0.01$); As compared with IG, the quantity of iIELs in small intestine of PG, MG and HG groups was significantly decreased ($P<0.01$), and that in LG group was decreased ($P<0.05$ or $P>0.05$). At 4 d, the quantity of iIELs in duodenum and ileum of IG was significantly higher than that in CG($P<0.01$); As compared with IG, the quantity of iIELs in duodenum and ileum of PG and HG groups were significantly reduced ($P<0.01$), and that in MG and LG groups were reduced ($P<0.05$ or $P>0.05$). However, at 4 d, the quantity of iIELs in jejunum of IG significantly lower than that in CG (P<0.01). The quantity of iIELs in jejunum of PG, LG, MG and HG groups were significantly increased as compare with IG ($P<0.01$) (Fig. 4).

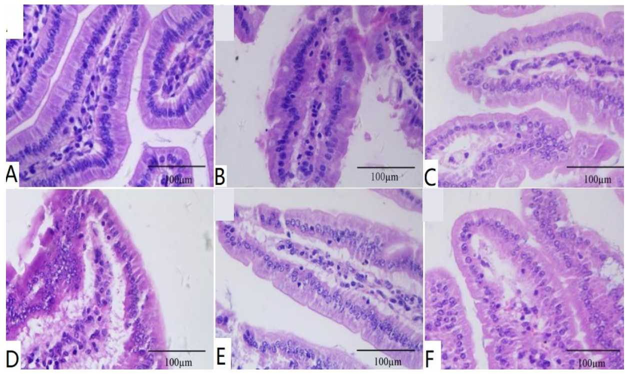

Fig. 3. Histological sections of duodenum of mice in each group (H and E analysis). Histological sections of intestinal epithelial cells revealed A, B, C, D, E, and F for CG, IG, PG, LG, MG, and HG groups respectively. 3A showed the normal structure; 3B showed the hyperplasia, flattened crypts, pyknosis and necrosis in the intestinal epithelial cells; 3C/D/E/F showed the recovered morphology of intestinal epithelial cells (scale bar 100 μm).

Table I. Primer sequence of *IL-6*, *TNF-α* and *GAPDH* mRNA.

Gene	primer sequence (5'-3')	Accession No.	Size (bp)
NM_001314054.1	*IL-6*-F	CTTCTTGGGACTGATGCTGGT	21
	IL-6-R	AGACAGGTCTGTTGGGAGTGG	22
NM_001278601.1	*TNF-α*-F	CAACGGCATGGATCTCAAAG	20
	TNF-α-R	TAGCAAATCGGCTGACGGT	19
XM_017321385.1	*GAPDH*-F	AGGCCGGTGCTGAGTATGTC	20
	GAPDH-R	GGCGGAGATGATGACCCTT	19

Table II. Incidence, protective rate and faecal score in diarrhea of infected-mice in 6 h.

Group	CG	IG	PG	LG	MG	HG
Incidence of diarrhea	0%	100%	30%	66.7%	60%	33.3%
Protective rate	——	0%	70%	33.3%	40%	66.7%
Faecal score	0.00±0.00^{A}	2.50±0.50^{C}	0.67±0.47^{AB}	1.17±0.37^{B}	1.00±0.58^{B}	0.83±0.37^{B}

Note: Different capital letters means highly significant difference between groups (P<0.01).

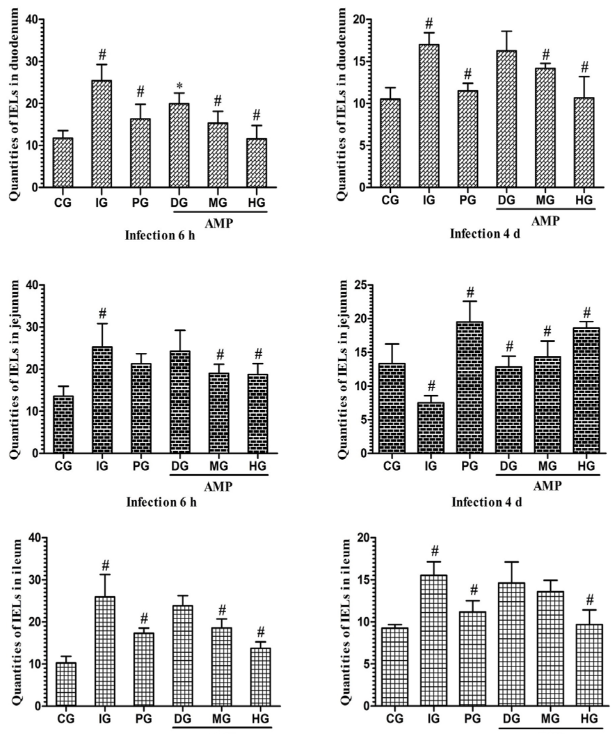

Fig. 4. Quantity of iIELs in the small intestine of mice at 6 h and 4 d.
The quantity of iIELs in the small intestine at 6h and 4d respectively. The results are expressed in arbitrary unit and quantity is compared from the control group. Symbols # and * indicates the P<0.01 and P<0.05 respectively in the small intestine.

Expression levels of IL-6 *and* TNF-α *in duodenum of mice*

The mRNA level of *IL-6* in duodenum of IG was significantly increased at 6 h and 4 d in comparison to CG respectively (P<0.01). However as compared with IG, the mRNA level of *IL-6* in duodenum of PG and HG gruops was decreased significantly (P<0.01). The mRNA level of *TNF-α* in duodenum of IG was significantly increased at 6 h and 4 d as compared to CG (P<0.01). Although the mRNA level of *TNF-α* in duodenum of PG and HG groups decreased significantly as compared with IG (P<0.01) (Fig. 5).

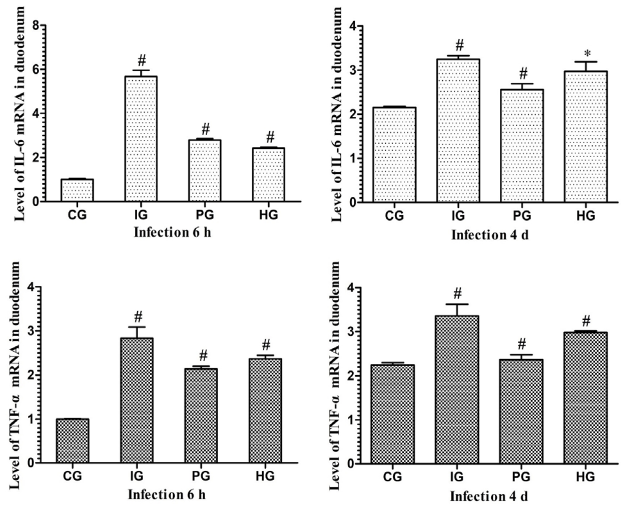

Fig. 5. The levels of *IL-6* and *TNF-α* mRNA in duodenum of mice at 6 h and 4 d.
Effect of AMP on IL-6, TNF-α mRNA Level in *E. coli* induced diarrheal mice was analyzed by reverse transcription qPCR. Results are expressed in arbitrary units; are shown relative to mRNA expression levels from the control group in duodenum of mice at 6 h and 4 d.

DISCUSSION

Intestinal intraepithelial lymphocytes iIELs are various populations of lymphoid cells that inhabit between the intestinal epithelial cells to form the intestinal mucosal barrier. The intestinal epithelial cells and iIELs are important components of the intestinal immune system against invading pathogen, they interface for immune cells to detect and respond to environmental substances (Suzuki, 2012). It is found that the excessive iIELs could reduce the functions of intestinal mucosal barrier (Hayday *et al*., 2001). However, the quantity of iIELs might be relative to the contact with the microbiota and pathogen types and dose of invading pathogen, also the functions of innate immune and mucosal immune response. The increase of iIELs was a characteristic index for early diagnosis of gastrointestinal diseases and tissues damages, which might be related to peptic duodenitis (Chang *et al*., 2005). Our findings revealed that the quantity of iIELs in small intestine of diarrheal mice was increased with *E. coli* induction suggested that *E. coli* might induce extensive enteritis in small intestine of the mice. The amount of iIELs in small intestine of diarrheal mice was decreased in dose-dependent manner with the pre-administration of AMP, which indicated that AMP could maintain the functions of intestinal mucosal barrier and as an anti-inflammation. The epithelial cells intestine proliferated rapidly to maintaining the steady state of intestinal mucosa, therefore the number of iIELs in diarrheal mice was relatively decreased. The pre-administration of AMP promoted the increase of iIELs in jejunum of diarrheal mice, which manifested that AMP maintained the functions of intestinal mucosal barrier by facilitating the proliferation of intestinal epithelial cells. The AMP regulates the disorder of intestinal flora, and improve the metabolism of animals (Wang *et al*., 2014). The effect of AMP on anti-pathogenic microorganism found better than antibiotics (Li *et al*., 2011), and it could promote lymphocyte proliferation after selenium modification (Liu *et al*., 2015). AMP possess biological activities to improve in digestive system, anti-tumor, anti-inflammatory, anti-aging, anti-oxidative, and anti-bacterial activities (Li *et al*., 2012, 2014; Shu *et al*., 2017; Wang *et al*., 2009, 2014; Kim *et al*., 2016).

IL-6 is considered a mediator of the inflammatory response and it has many biological functions induced by iIELs, which can protect the colon from invasion by pathogen and the integrity of intestinal mucosa (Kuhn *et al*., 2018). IL-6 acts on pro-inflammation, stimulates the inflammatory injury and the development of tumors when largely proliferation (Fizazi *et al*., 2012). The iIELs of $TCR\beta^{+}CD4^{-}CD8^{-}$ in colon of mice could produce a large number of IL-6 in the early stage of inflammatory injury (Kuhn *et al*., 2014). The present study showed that the expression of *IL-6* in the duodenum of diarrheal mice was significantly higher than those normal mice. The pre-administration of AMP decreased the mRNA transcription of *IL-6* in the duodenum of diarrheal mice; our results were consistent with other previous research (Frech and Hudson, 2015; Herlina *et al*., 2016; Pei *et al*., 2019; Zhou *et al*., 2006) as well. The study suggested that AMP effectively antagonize duodenitis and diarrhea in mice and protective effects in intestinal cells and tissues.

The production of inflammatory mediators is cascade process, and the NF-κB signaling pathway is a classical pathway that induces inflammatory mediators. The study found that TNF-α could be involved in the activation of NF-κB inflammatory signaling pathway, which ultimately activates the inflammatory cells to express more inflammatory factors such as IL-6, TNF-α, etc. (Kuhn *et al*., 2018). Many diseases are associated with excessive secretion of inflammatory mediators (Tsoulfas and Geller, 2001). The anti-TNF-α therapy could effectively cure rheumatoid arthritis and prove that TNF-α playing an important role in the development of inflammation (Feldmann and Maini, 2001). TNF-α highly expressed due to the stimulating ROS production in intestinal epithelial cell (Rokutan *et al*., 2008), which could induce the chronic inflammatory bowel disease (Ahn and Kim, 2018). Our study shows that the transcription of *TNF-α* in duodenum of diarrhea mice was increased, suggested that the inflammatory response of duodenum in diarrhea mice might relate to the activation of NF-κB inflammatory signaling pathway. However, the pre-administration of AMP reduced transcription level of *TNF-α* in diarrhea mice, which was consistent with the previous results (Belluzzo *et al*., 2014; Park *et al*., 2017; Yang *et al*., 2005). The anti-inflammatory mechanism might be related to the inhibition of NF-κB inflammatory signaling pathway activation.

CONCLUSION

In conclusion, the results reported here show that AMP is a potential alternative approach for treating diarrhea condition by regulating the quantity of iIELs in small intestine. The anti-inflammatory effect of AMP is associated with the down-regulation of the *IL-6*, *TNF-α* transcriptional level and the balanced pro-inflammatory cytokine in diarrheal mice.

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Conflict of interest statement

The authors have declared no conflict of interest.

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