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PROTECTIVE EFFECTS OF TOTAL POLYSACCHARIDES IN COMPOUND KUQININ CANINE PARVOVIRUS INFECTION AND ITS INFLUENCE ON THE EXPRESSION OF *BAX* AND *BCL-2* GENE BASED ON PATHOLOGICAL, ULTRASTRUCTURAL, AND APOPTOTIC CHANGES

L. Yi-Chen, Z. Zhao-Rong, L. Juan* and G. Zhi-Sheng

Laboratory of Chinese Herbal Drugs Innovation Research (Room 3611), South-west University, Rongchang campus, Rongchang, Chongqing, China, 402460
*Corresponding Author e-mail: liujrc@163.com.

ABSTRACT

As one of the most dangerous infectious diseases to dogs, canine parvovirus (CPV) infection has been universally characterized by its short course, high incidence, strong infectivity, and high mortality rate. This study was conducted to observe the protective effects of total polysaccharides (TP) found in Compound Kuqin (CK) on parvovirus infected dogs, and the expression levels of *Bax* and *Bcl*-2 gene in small intestine were detected as main index. Compound Kuqin (CK) is composed of a variety of Chinese herbal medicine, including *Radix Astragali, Radix Scutellariae*, *Flos Lonicerae*, and so on. Fifty dogs were equally divided into five groups, including the CK, TP, positive, blank, and model groups. The blank group was injected intramuscularly with normal saline for 7 d, and the *Astragalus* polysaccharides were used as the positive control. For CK, and TP groups, all the experimental dogs were intramuscularly injected with CK solution, and TP solution (1.5 mL/Kg body weight) twice daily for 7 d, respectively, and challenged by CPV post-vaccination. For the model group, the dogs were challenged by CPV without treatment. Small intestinal tissues were collected from the experimental dogs, and were further studied by transmission electron microscopy. The apoptosis of small intestinal cells, and the expression levels of *Bax* and *Bcl-2* genes and proteins were detected using TUNEL assay, real-time fluorescent quantitative PCR, and western blot, respectively. The results indicated that in the model group, the intestinal villi were desquamated, the epithelium cells were degenerated, and the rate of apoptosis was 11.45±1.70%, the expression levels of *Bax*, *Bcl*-2 mRNA and protein was 3.55, 0.45, 0.46, and 0.52, respectively, and the ratio of *Bcl*-2/*Bax* was 1.15. However, the intestinal villi of dogs in TP group were only slightly desquamated, the epithelium cells were also slightly degenerated, villi boundaries were clear, the rate of apoptosis decreased significantly ($P \leq 0.01$), the expression of *Bax* mRNA and protein decreased significantly ($P \leq 0.01$ or $P \leq 0.05$), and the expression of *Bcl*-2 and the ratio of *Bcl*-2/*Bax* increased significantly ($P \leq 0.01$).It showed that Total Polysaccharides in Compound Kuqin could protect the small intestinal tissue, and promote the recovery of the small intestinal tissue by inhibiting ultrastructural changes in the small intestinal lining in parvovirus infected dogs as well as reducing the cell susceptibility to apoptosis induced by Canine parvovirus.

Keywords: Polysaccharides in Compound Kuqin, Canine parvovirus, small intestinal tissue, *Bax*, and *Bcl*-2, protective effect

INTRODUCTION

Canine parvovirus (CPV) infection is a highly contagious disease primarily affecting dogs of all ages with the clinical symptoms of severe vomiting, hemorrhagic enteritis, leucopenia, and myocarditis. Its infection rate can be as high as 100%, and its mortality rate ranges from 10% to 50%, sometimes up to 100%. In recent years, researchers had carried out a series of studies on the prevention and treatment of CPV infection, and obtained some achievements. They developed the CPV nucleic acid vaccine, CPV recombinant live vector vaccine, CPV enteritis attenuated vaccine, and CPV monoclonal antibodies. However, since CPV itself can easily generate new mutations by antigenic drift, resulting in host rang expansion, and the interference of maternal antibody, the second infection and environmental factors, which could only lead to poor clinical out come of the vaccination. However, researchers found that some polysaccharides of traditional Chinese medicine can effectively prevent and treat the CPV caused disease, which provides a new research direction for controlling the CPV infection. In-vitro and in-vivo studies confirmed that CPV can induce apoptosis and necrosis of the intestinal mucosa with secondary infection (Bauder *et al*., 2000; Nykky *et al*., 2010). Intestinal cell apoptosis induced by CPV infection can be regulated by a variety of apoptosis-related genes and cytokines. Among them, Bcl-2 and Bax are the two representative members of the Bcl-2 family and are the key regulators of apoptosis in the mammalian cell. These genes play regulatory action in the dimer form of homologous or heterologous by

inter-conjugation (Liu *et al*., 2010; He *et al*., 2012). The prescription was studied out by our group according to the theory of Traditional Chinese Veterinary. Many herbal recipes, like Radix Astragali, Radix Scutellariae, Radix Sophorae Flavescentis, and Flos Lonicerae have the effects of clearing away heat and dry dampness, stopping diarrhea, and detoxication, where polysaccharide is one of the essential components in these compounds (Rincão *et al*., 2012; Song *et al*., 2013). According to the modern research progress concerning ph armacological effect, Chinese herbal polysaccharide has the effects of immune-regulation, anti-cancer, anti-inflammatory, and anti-virus (Ma *et al*., 2013; Tseng *et al*., 2016; Ayeka *et al*., 2016). Compound Kuqin has the efficiency of regulating qi, invigorating spleen, promoting diuresis, removing dampness, clearing away heat and toxins, and arresting dysentery. And the Kuqin is composed of light yellow Sophora root, Baikal Skullcap root, Chinese Pulsatilla root, Honeysuckle flower, and Membranous Milk vetch root, etc. Although our previous study has demonstrated that Compound Kuqin could effectively prevent and treat CPV infection by enhancing body immune system and protecting the gastrointestinal mucosa from injury (Li *et al*., 2010; Zhu *et al*., 2010, 2012 and 2013; Du *et al*., 2012; Liu *et al*., 2013), yet the mechanism remains unclear. In the present investigation, the mechanism of preventing CPV disease by total polysaccharides extracted from Compound Kuqin was studied based on the successful establishment of canine models of parvovirus enteritis. This would provide a theoretical basis for the clinical application of Compound Kuqin for preventing CPV disease.

MATERIALS AND METHODS

Virus strain and cells: CPV strain was provided by Prof. Peng G.N., Sichuan Agricultural University. Primary feline fetal kidney cells (F81 cell) were obtained from the Center for Cell Resources of Shanghai Institute, Chinese Academy of Sciences.

Experimental Animals: The experiment was conducted on 50 healthy Chinese rural dogs (2 to 4 months old) weighing 2.0 ~ 4.0 Kg, keeping in the Experimental Animal Department in College of Animal Science, Southwest University. Prior to the experiment, all the experimental dogs were injected with Levamisole to eliminate parasites and quarantined for 10 days.

Drugs and Reagents: Extracting solution and total polysaccharides extracted from Compound Kuqin with the same concentration of 0.04 g/mL were provided by Traditional Chinese Veterinary Medicine Innovation Laboratory Research Center in College of Animal Science, Southwest University. The extraction was provided by College of Animal Science, Southwest University and the method of extraction is thoroughly described in previous study (Zhu *et al*., 2013). First, the whole medicinal materials were put into the petroleum ether and 80% ethanol to remove impurity by the method of heating reflux. The filtrate was extracted twice in boiling water after volatilizing the solvent. The extracting solution was concentrated and precipitated by alcohol. The protein in sediment was removed by adding sevage reagent after high-speed centrifugation. The total polysaccharide was prepared after washing with ethanol, acetone, diethyl ether, in turn, precipitating and drying (Pan *et al.*, 2014; Pu *et al*., 2014).

Experimental Design: The experiment was conducted on Fifty healthy Chinese rural dogs, which were randomly divided into 5 groups, including the blank group, the model group, the Compound Kuqin (CK) group, the total polysaccharide extracted from Compound Kuqin (TP) group, and the positive drug (*Astragalus* polysaccharides, APS) group. For the model group, the dogs were artificially challenged with 2 mL of CPV solution per dog with the TCID50 of 10-5.76/0.1 mL (Du *et al*., 2012; Lu, 2012). For other treated groups, the dogs were intramuscularly injected with extracting solution from Compound Kuqin, total polysaccharides extracted from CK and *Astragalus* polysaccharides solution for continuous treatment with 1.5 mL/Kg Bodyweight and twice daily for 7 d before challenged by CPV (Lai *et al.*, 2014). The blank group was injected intramuscularly with physiological saline 1.5 mL/Kg bodyweight for 7 d without CPV challenge.

Study of Cell Structure of Intestinal Mucosa: Paraffin slices of intestinal tissue taken from the same segment in the all experimental dogs were prepared and observed by light microscope OLYMPUS CX31according to the methods described by Yang (2006) and Lv *et al*. (2008).

Study of Cell Ultrastructure of Intestinal Mucosa by Electron Microscopy: Electron microscopic slices of the intestinal tissues taken from the same segment in the all experimental dogs were prepared and observed by JEM-1400 transmission electron microscopy according to the methods described by Du *et al*. (2012) and Wu *et al*., (2004).

Detection of Cell Apoptosis by TUNEL assay: The small intestine tissue cells form of paraffin embedding slice prepared in procedure 6 were strictly detected by TUNEL apoptosis detection kit bought from Nanjing Jiancheng Bioengineering Institute, China(Liu and Sun, 2005). Blue and brown nuclei were observed under the optical microscope, normal and apoptotic cells appearing blue and brown nuclei in color, respectively. The total normal and apoptotic cells were counted based on taking 5 high power fields (at least 100 or more cells). The cell apoptosis rate was calculated according to the following formula:

Cell apoptosis rate = the number of apoptosis

cells in view/ the total number of cells of computing in the field of vision×100%.

Study of *Bax* and *Bcl*-2mRNA Expression

Total RNA Extraction and Identification from Small Intestine Tissue and cDNA Synthesis: Total RNA from small intestine tissue was extracted according to RNAiso TMPlus extraction kit instruction, whichproduced by TaKaRa Bioengineering (DaLian).Co., Ltd The quality of mRNA was qualified by the ratio of the reading at 260 nm and 280 nm (A260/A280) and 1.5% agarose gel electrophoresis described by Liu *et al*. (2013). The cDNA was synthesized according to PrimeScript RT reagent Kit with gDNA Eraser instruction, which produced by

Primers Design TaKaRa Bioengineering (DaLia n) Co., Ltd and stored at -20℃ before using.

All the specific real-time PCR primers of *Bax, Bcl-2* and *β-actin* were designed with the aids of Primer Premier 5.0 and Oligo softwares. The sequences of primers are shown in table 1, which were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co.

Table1. Primers used in this study

Genes	Primers (special forward primer, SF; special reverse primer, SR)	Length of product (bp)	Accession No.
Bax	SF:5'-CAGAAAACATTTCAGCCGCCACTC-3'	234	XM-003585203.1
	SR:5'-CCTTTTGCTTCAGGGTTTCATCCAG-3'		
Bcl-2	SF:5'-CTGAGTACCTGAACCGGCATCT-3'	171	NM-001002949.1
	SR:5'-GAGACAGCCAGGAGAAGTCAAAC-3'		
β-actin	SF:5'-AGCGGGAAATCGTGCGTG-3'	179	XM-544346.1
	SR:5'-CAGGGTACATGGTGGTGCC-3'		

RT-PCR Amplification of *Bax*, *Bcl*-2 and β-Actin Gene: RNA extracted from small intestine tissue was reverse-transcribed into cDNA according to PrimeScript RT reagent Kit With gDNA Eraser instruction manual, and the objective band was observed after PCR amplification.

Quantitative Real-time PCR Amplification of *Bax*, *Bcl*-2 and β-Actin Gene in Dog: The concentration of PCR amplification products of *Bax*, *Bcl*-2 and *β-actin* genes were detected by the ultraviolet spectrophotometer made by General Analysis of General Instrument Co., Ltd. Beijing, China. The amplification products were used as template after gradient-diluting ten times for real-time fluorescent quantitative PCR amplification in order to establish the standard curve. The test results of *Bax*, *Bcl*-2 and *β-acti*n genes were calculated by Bio-Rad CFX Manager 1.6 software according to their own standard curve. The specificity of amplification products was detected by the solubility curve. *Bax* and *Bcl*-2 gene expression intensities were represented by the ratio of their absolute copy number to β-actin absolute copy number to correct the effect of RNA quantitative, reverse transcription and PCR reaction. All the results were transferred into relative fold expression, namely QR=2-△△Ct. Thus, the result was the multiple relationships of *Bax* and *Bcl*-2 gene between the experimental and the control group.

***Bax* and *Bcl*-2 protein expression:** One hundred milligrams intestinal tissue obtained from all the experimental dogs was grinded into liquid form, and the supernatant was separated by centrifugation for 10 min at 12,000 RPM in 4℃. The concentration of protein in cell lysis supernatant was evaluated according to BCA Protein Assay Kit manual【(1)Preparation of Standards and Working Reagent (required for both assay procedures) A. **Preparation of Diluted Albumin (BSA) Standards**

Preparation of the BCA Working Reagent (WR):

A. Use the following formula to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample: (9 standards + 3 unknowns) × (2 replicates) × (2mL) = 48mL WR required Note: 2.0mL of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

B. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B. Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days

when stored in a closed container at room temperature (RT)
(2). Procedure Summary (Test-tube Procedure, Standard Protocol)
(3). Test-tube Procedure (Sample to WR ratio = 1:20)
(4). Microplate Procedure (Sample to WR ratio = 1:8)】 produced by TaKaRa Bioengineering (DaLian) Co., Ltd. The proteins through 10% SDS-PAGE electrophoresis separation were successfully transferred onto PVDF membrane, and stained with Ponceau S solution. X-ray film was used for PVDF membranes developing and fixing after washing. The band intensity of protein was analyzed by American UVP gel imaging system using GAPDH as an internal reference. The ratio means the relative content of this protein.

Statistical analysis: SPSS 19.0 software was used for statistical analysis and all the experimental data was indicated with mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used for the comparison among all the different groups and a $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Pathological Observations: Compared with the blank group, the results indicated that in the positive drug group, the small intestinal villi were sloughed, and some of which even disaggregated. The margin of the villi was not obvious, meanwhile edema within lamina propria under villi and intrinsic morphous was unclear. Epithelial cell denatured, central lacteals disappeared, and a large number of leukocytes infiltrated in themodel group through light microscope observation. Epithelial cells were slightly degenerated, the villi atrophied with distinct margin. In CK group, only a few villi were sloughed off with desquamated epithelial cells and the majority of villi were intact with normal histological appearance. In the TP group, the small intestinal villi were partly sloughed and mildly atrophied and epithelial cell lightly denatured. This suggested that the pathological change was markedly reduced in the treated group compared with the model group. The findings of this study are presented in Fig. 1.

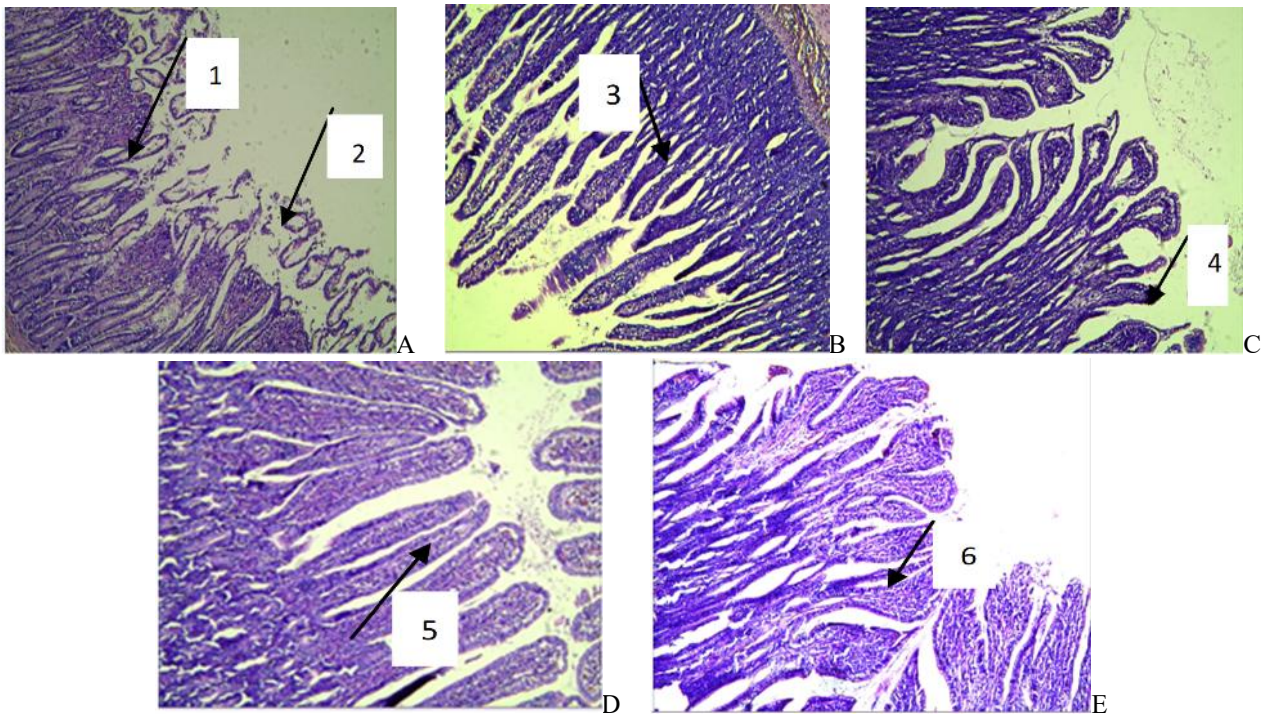

Fig.1 Histopathological observations in the experimental dogs of each group H.E.（10×）

A. model group: 1 villi edema, 2 villi sloughing, B. blank group: 3 normal small intestinal villi; C. positive drug group: 4 a little villi appears necrotic; D and E: 5 and 6 the changes of villi in CK group and TP group.

Ultrastructural / Electron Microscopy Studies: The results through transmission electron microscope observation indicated that in the blank group,the microvilli in the epithelium of small intestinal were integrated and distinct, which were lining up in orderwith clearcell margin, nucleus integrated with abundant mitochondria, endoplasmic reticulum and goblet cells, and no cells sloughed. Inthe model group, microvilli in the epithelium of small intestine were sloughed and

water-structure emerged, mitochondria of epithelial cells stretched, endochylema dissolved and partly collapsed, cell edge metachromatic stained and centralized to one side, partial nucleus swelling and karyorrhexis, and the inter space between cells were obvious. In the positive drug group, microvilli shorted membrane of goblet cells damaged, microvilli in the epithelium slightly atrophied and partly necrosed. InCK and TP groups, there were a few microvilli sloughed but with whole intrinsic structure and distinct intrinsic morphous, nuclear membrane integrity, have normal cell-cell junction, which showed the ultrastructures were improved markedly after treatment. The results are illustrated in Fig. 2.

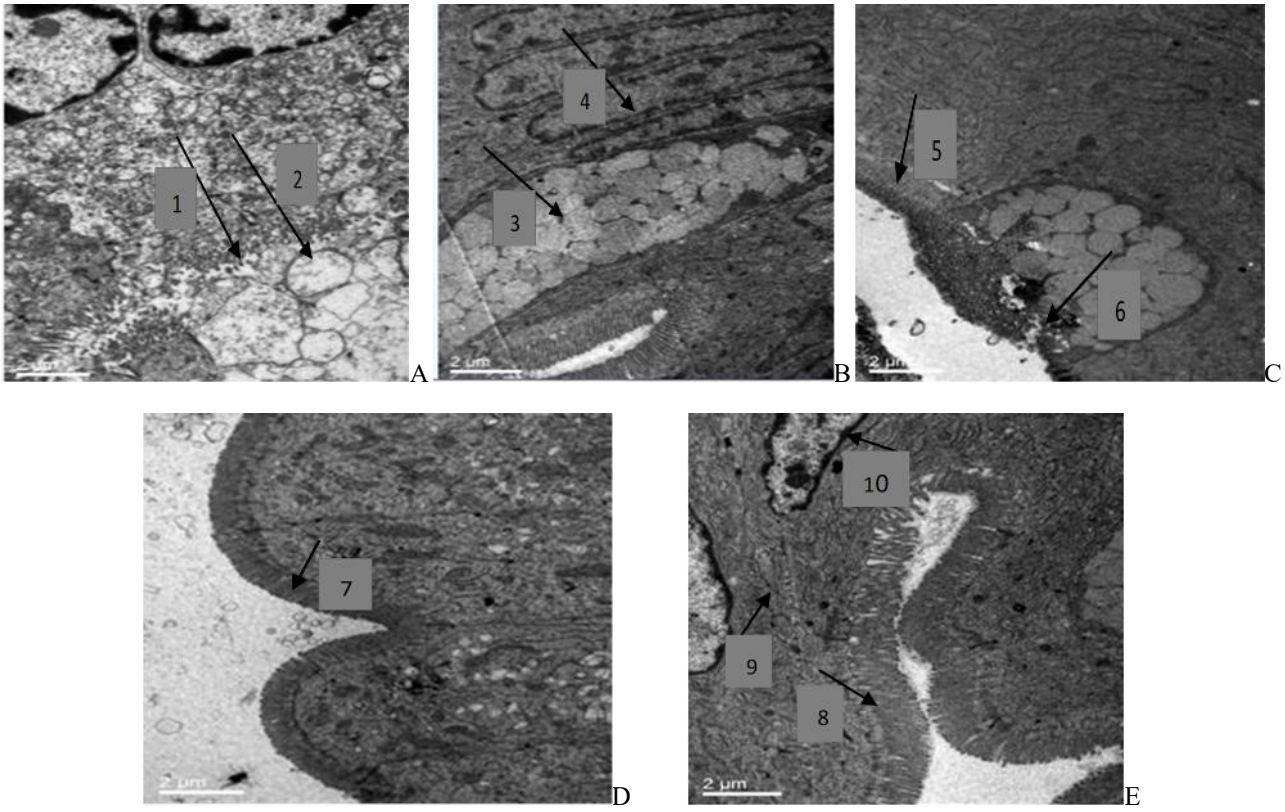

Fig.2 Ultrastructural changes in intestine of the experimental dogs of each group

(A) The model group with microvilli sloughing and mitochondria stretching, marked with 1 and 2 respectively; (B) Blank group with normal goblet cells and intestinal villi, marked with 3 and 4 respectively; (C) Positive drug group with microvilli shortening and membrane of goblet cells damaged, marked with 5 and 6 respectively; D and E were for CK group and TP group with integral microvilli, marked with 7 and 8, normal cell-cell junction and nuclear membrane, marked with 9 and 10, respectively.

Cell Apoptosis Studies: In the model group, there were a great quantitative numbers of pycnotic and apoptotic cells with concentrating nucleus which were deeply stained by brown-yellow color. The rate of cell apoptosis was 11.45 ± 1.70%, which was significantly higher than that of the blank group (1.65 ± 0.73%). The rates of cell apoptosis decreased significantly ($P \leq 0.01$) after intramuscular injection of APS, extracting solution from CK and total polysaccharides extracted from CK and the apoptosis rates were 3.22±0.63%, 3.06±0.94% and 3.31±1.35%, respectively. The results are presented inFig. 3 and Table 2.

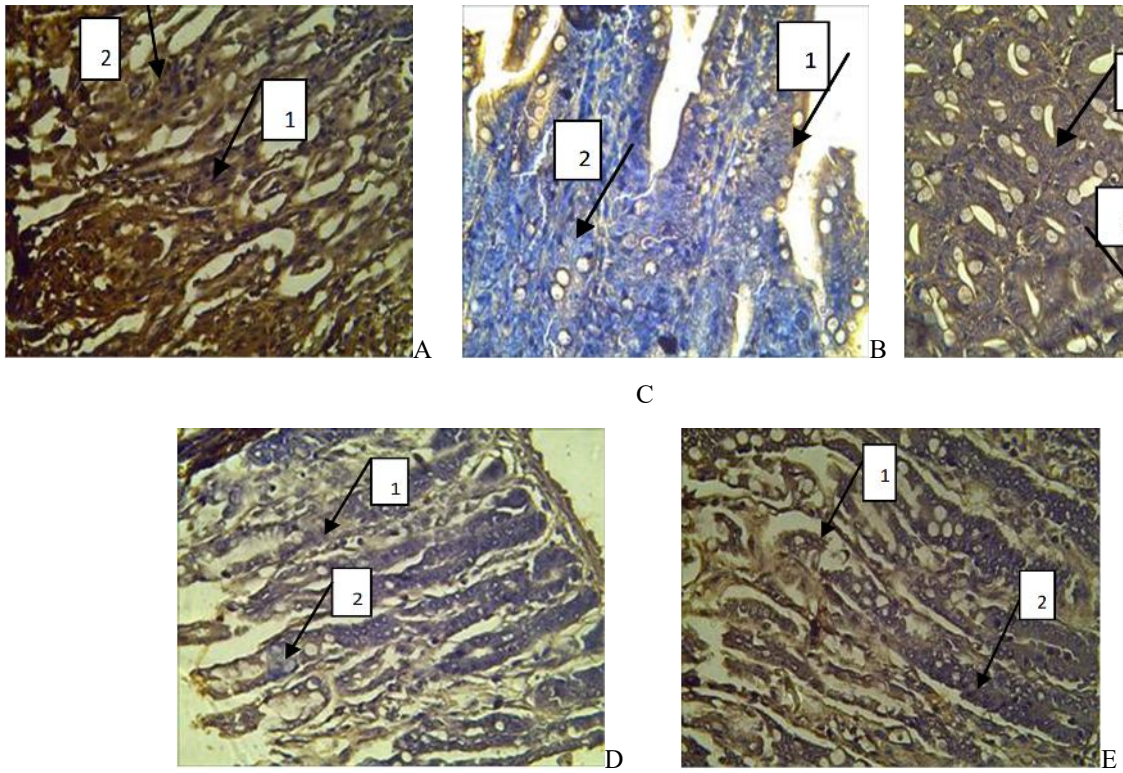

Fig.3 Ultrastructural observations in the experimental dogs of each group TUNEL（40×）

(A) model group; (B) blank group; (C) positive drug group; (D) CK group; (E) TP group. apoptotic nuclei with brown-yellow (1) and normal nucleus with blue color (2).

Table 2. The cell apoptosis rate of small intestine tissue in each group (unit:%).

Group	Number of animals	Intestinal apoptosis rate
blank group	10	1.65±0.73**
model group	10	11.45±1.70
positive drug group	10	3.22±0.63**
CK group	10	3.06±0.94**
TP group	10	3.31±1.35**

Note: * and ** Data differ when compared with the model group after intramuscularly injecting different drugs ($P \leq 0.05$) or ($P \leq 0.01$).

The Effect of Total Polysaccharide Extracted from Compound Kuqin on mRNA Expression of *Bax* and *Bcl*-2 Genes in the Small Intestinal Tissues

Fluorescent Quantitative Real-time PCR Standard Curve: The fluorescent quantitative real-time PCR standard curves of *Bax*, *Bcl-2*, and *β-actin* genes were shown in Fig. 4 with the regression coefficient of 0.997, 0.999, and 0.997 after 10 folds serially diluted, respectively. The calibration curves showed excellent linearity.

Effect on *Bax* mRNA Expression: The results of the fluorescent quantitative real-time PCR showed that the expression level of *Bax* mRNA in small intestinal tissue significantly decreased by 80.69% after intramuscularly injection with total polysaccharide extracted from CK compared with the model group. There were also significant differences between other groups ($P \leq 0.01$) (Fig. 5).

Effect on *Bcl*-2 mRNA Expression: As can be seen in Fig. 9, the fluorescent quantitative real-time PCR result showed that the expression level of *Bcl*-2 mRNA in small intestinal tissue significantly increased by 1.61 times after intramuscular injection with total polysaccharide extracted from CK compared with the model group. And there were also statistically significant differences between other groups ($P \leq 0.01$).

Effect on Expression of *Bcl*-2/*Bax* mRNA: The mRNA expression ratio of *Bcl*-2 to *Bax* gene in the model group was decreased compared with the blank group ($P \leq 0.01$) (Fig. 10). The mRNA expression ratio of *Bcl*-2 to *Bax* gene in small intestinal tissue was significantly increased by 8.35 times after intramuscularly injection with total polysaccharide extracted from CK compared with the model group ($P \leq 0.01$).

Western blot showed that the expression level of

Bax protein in small intestinal tissue in the model group was significantly increased compared with the blank group ($P \leq 0.01$). On the contrary, *Bcl*-2 protein expression and the expression ratio of *Bcl*-2 to *Bax* were both significantly decreased ($P \leq 0.05$). Likewise, *Bcl*-2 protein expression and the expression ratio of *Bcl*-2 to *Bax* in small intestinal tissue of all the experimental dogs in TP group were significantly increased, whereas, the expression of *Bax* protein was significantly decreased compared with the model group ($P \leq 0.01$).

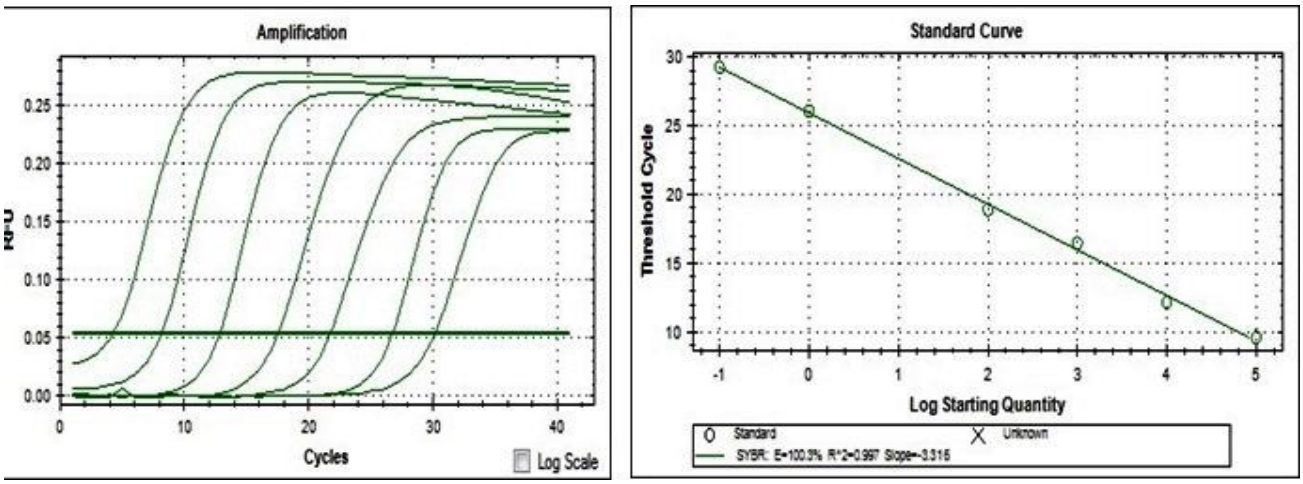

Fig.5. The fluorescent quantitative real-time PCR standard curve of *Bax* gene in canine intestine tissue.

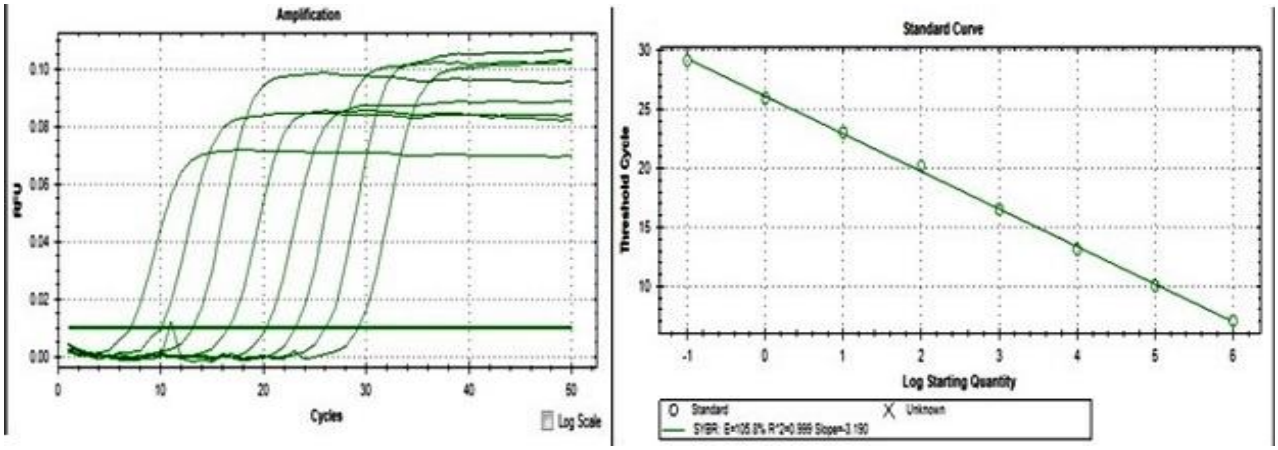

Fig.6. The fluorescent quantitative real-time PCR standard curve of *Bcl*-2 gene in canine intestine tissue.

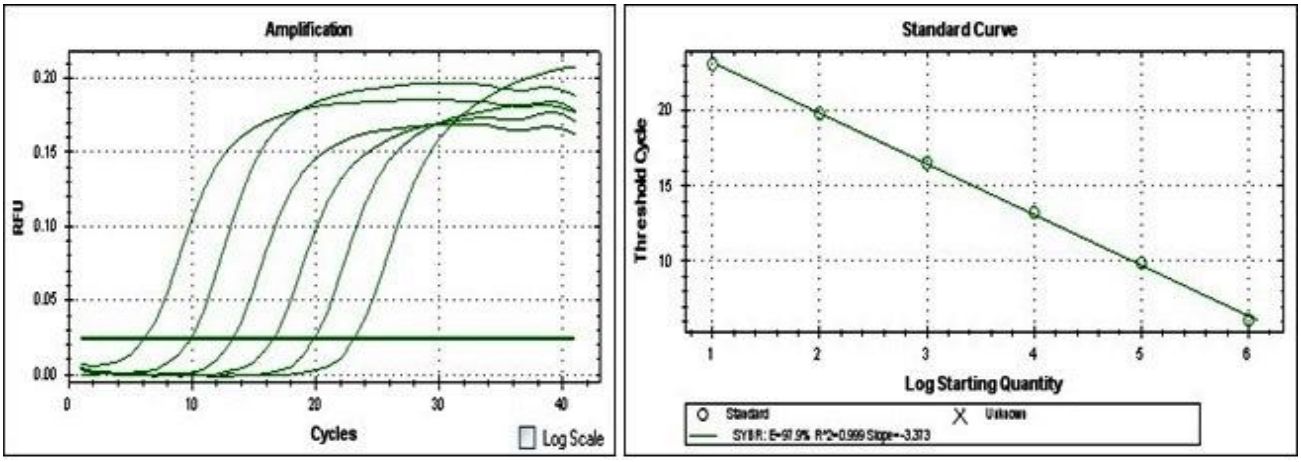

Fig.7. The fluorescent quantitative real-time PCR standard curve of β-*actin* gene in canine intestine tissue

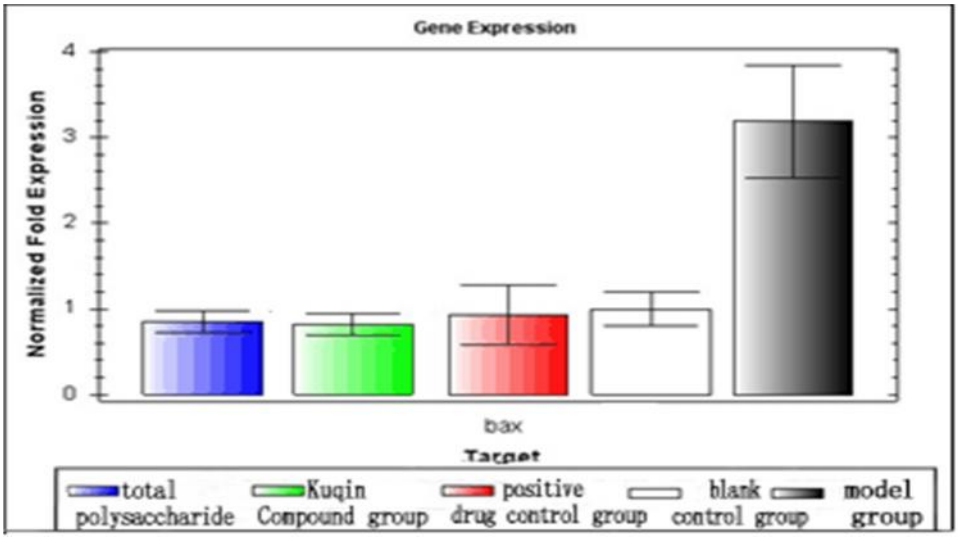

Fig.8. Relative expression level of *Bax* mRNA in canine small intestine tissue in each group (*n*=10)

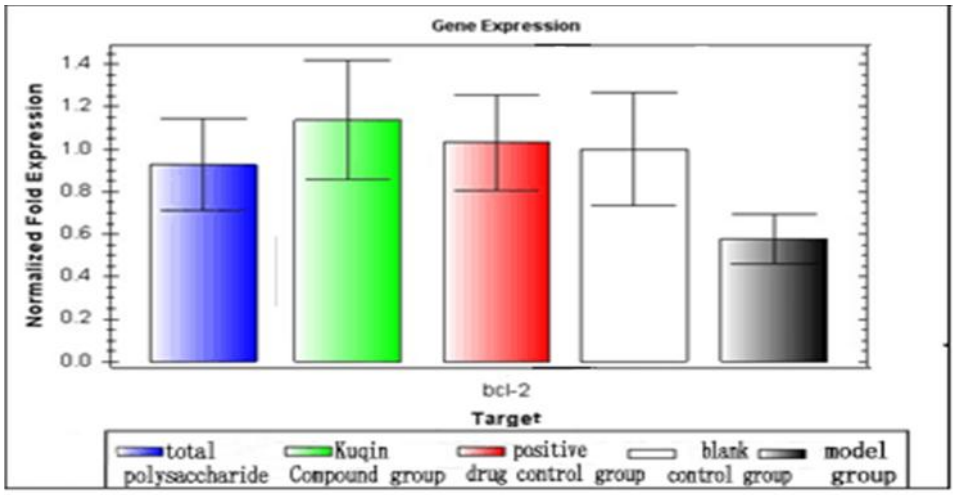

Fig.9. Relative expression level of *Bcl*-2 mRNA in canine small intestine tissue in each group(*n*=10)

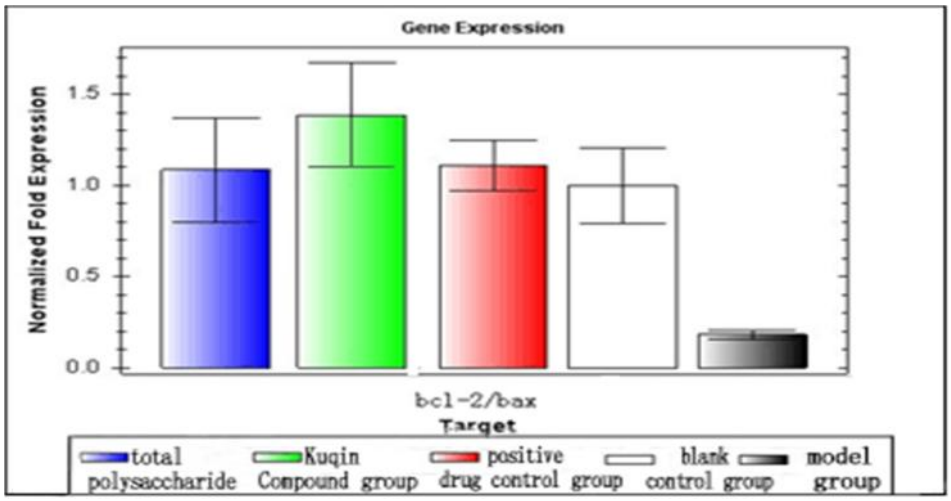

Fig.10. Expression ratio of *Bcl*-2/*Bax* mRNA in canine small intestine tissue in each group (*n*=10)

Effects on *Bax*, *Bcl*-2 Protein Expression studies by Western blotting

Table3. Expression of *Bax* and *Bcl*-2 proteins in canine small intestine tissue in each group (*n*=10).

Group	Bcl-2/GAPDH（%）	Bax/GAPDH（%）	Bcl-2/Bax（%）
Model group	0.52	0.45	1.15
Blank group	0.54	0.4	1.35
Positive drug group	0.55	0.46	1.21
CK group	0.68	0.40	1.72
TP group	0.59	0.32	1.83

DISCUSSION

According to the major clinical manifestations, CPV disease is divided into two types i.e. myocarditis type and enteritis type（DU Lin-Lin,2012）. In the enteritis type, dogs infected with CPV show symptoms including vomiting and bloody diarrhea, etc (Vera and Andrea, 2013). By the theories of syndrome differentiation in Chinese Veterinary Medicine, CPV disease belongs to damp-heat diarrhea due to stagnation of the pathogenic dampness and heat-toxin in stomach and intestine. For treatment, it is advisable to clear away heat, dry dampness, and arrest dysentery. According to the disorder of this syndrome, we self-prescribed Compound Kuqin which was composed of *Baikal Skullcap Root*, *lightyellow Sophora root*, *Largehead Atractylodes Rhizom*e, *Honeysuckle flower*, and *Membranous Milkvetch root*, etc. *Baikal Skullcap Root* has the effect of clearing away heat, drying dampness, purging fire and removing toxic materials, which serves mainly to treat epidemic febrile diseases, upper respiratory tract infection, jaundice due to damp-heat and diarrhea. *Lightyellow Sophora Root* is considered favorable in clearing away heat and expelling dampness for the treatment of damp-heat diarrhea.*Honeysuckle Flowe*r serves to clear away heat and toxic materials and expel wind-heat from the body surface which is usually applied to treat summer-heat syndrome, diarrhea and influenza. According to the prescription-composing characters, Compound Kuqin has the effect of regulating *chi*, invigorating spleen, promoting diuresis, removing dampness, clearing away heat and toxic materials and arresting dysentery. Our previous study had proved that Compound Kuqin showed significant effect on the prevention and treatment of CPV disease.

Polysaccharideis one kind of important components in Chinese herbal medicine. Recently, studies have shown that polysaccharides extracted from herbal medicine exhibit excellent effects against virus, tumor, and regulating immunity. (Wasser, 2002; Jiang *et al*., 2010; Liu *et al*., 2013; Areca *et al*., 2016). As tragalus polysaccharide is one of the components in Chinese herbal medicine, currently widely used as anti-viral reagent; the main mechanism of anti-influenza virus may be related to stabilize the cell membrane and enhance the resistant ability of cells. In this research, light microscope and transmission electron microscope were used for observing the structure of small intestine based on successfully establishing the CPV of enteritis type. The results indicated that total polysaccharide extracted from CK had protective effect on the injury of structure and ultra-structure of small intestinal tissue. It effectively prevented sloughing and denaturating of villi and microvilli in the small intestine with clear and intact villi margin and intrinsic morphology. It can also preventcell from denaturating, making cell membrane integrating and normal conjunction of cell margins in order to protect cells' normal morphous in intestinal tissue. Thereby, total polysaccharide extracted from CK can improve the injury of small intestinal tissue due to CPV through stabilizing the structure of cells. The appearance of this effect is achieved by inhibiting virus proliferation and reducing virus binding ability to the cells (Zuo *et al*., 2013; Hu *et al*., 2015; Liu *et al*., 2015).

Cell apoptosis is also known as Programmed Cell Death (PCD). The family of *Bcl*-2 gene was the main factor which played a major role in the regulation of apoptosis. The expression of *Bax*/*Bcl*-2 heterodimers was the important link for regulating the process of cell apoptosis. The ratio of *Bcl*-2/*Bax* decided the regulating direction of apoptosis. (Ou *et al*., 2008). The TUNEL (**Terminal dexynucleotidyl transferase (TdT)-mediated dUT Pnickend labeling**) kit was used for observing the cell apoptosis inthe small intestinal tissue of the dogs infected by CPV, and the results showed that cell apoptosis in small intestinal tissuewas significantly decreased after intramuscularly injection with total polysaccharide extracted from Compound Kuqin ($P \leq 0.01$). In the model group, mRNA transcription and protein expression of anti-apoptosis gene *Bcl*-2 in small intestinal tissue were inhibited since mRNA transcription and protein expression of gene *Bax* increased and the ratio of *Bcl*-2/*Bax* significantly decreased. These results were consistent with conclusions drawn by other reports of cell apoptosis increased induced by virus infection in intestinal tissue (Schäbitz *et al.*, 2000).

We also found that total polysaccharide extracted from Compound Kuqin could inhibit the cell apoptosis in intestine by increasing mRNA transcription and protein expression of *Bcl*-2, which accompanied with

significantly decreasing in mRNA transcription and protein expression of gene *Bax* and the increase of the ratio of *Bcl*-2/*Bax* in canine small intestinal tissue. Thus, Compound Kuqin and total polysaccharide extraction could inhibit the cell apoptosis through increasing the ratio of *Bcl*-2/*Bax*, conjugation of *Bcl*-2/*Bax* homodimer and mitochondrial endomembrane to inhibit the changes in the permeability of mitochondria and affect the formation of macro-hole according to the mechanism of apoptosis (Watt *et al*., 2008; Clark *et al*., 2006).

Meanwhile, Compound Kuqin and total polysaccharide extraction could protect the normal morphology of cells in canine small intestinal tissues from CPV infection. The results implicated that Compound Kuqin and total polysaccharide extraction may also havethe protective effects on the morphology and permeability of mitochondria. Cell apoptosis was inhibited possibly due to the stabilization of the permeability of mitochondria inhibited cascade pathway. But the underling mechanism of action remains uncertain and further study needs to be carried out to figure out the exact mode of action of these compounds (Pang *et al.,*2018).

Conclusions: This study provides evidence that total polysaccharide extracted from Compound Kuqin can effectively prevent the intestinal tissue from injury that caused by CPV infection, and could offer better effects in healing and repair of small intestinal tissue damage. The results demonstrated asuccessful approach in the direction of new antiviral drug discovery from indigenous herbal plant that is Compound Kuqin.

Authors' contributions: ZZR and LJ conceived and designed the study. GZS and LYC performed the experiments and collected the data. LYC wrote the manuscript. All authors read and approved the final manuscript.

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