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RESEARCH ARTICLE

Effect of Kuqin Compound Total Polysaccharides on $CD4^+$, $CD8^+$ T Cells and Cytokines of Blood in Dogs Infected by Canine Parvovirus

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ABSTRACT

With the purpose of researching on the effect of Compound Kuqin total polysaccharides on T cell subsets and cytokines of blood in canines with canine parvovirus, the model was established, and 120 canines were separated into 6 groups, such as blank control group (Group Ⅰ), model group (Group Ⅱ), positive medicine group (Group Ⅲ), compound Kuqin total polysaccharides high dose group (Group Ⅳ), middle dose group (Group Ⅴ), low dose group (Group Ⅵ). We set the time point when the virus was injected into canines for 6 hours as day 0, and collected the blood sampling from the canine saphenous vein at different time. Finally, we detected the percentages of T lymphocyte subsets and the content of IL-2, IL-4 and IFN-γ. Compared with Group Ⅰ, the percentage of $CD3^+CD4^+$ T cells in Group Ⅱ decreased in 0-35d, $CD3^+$ T cells decreased and $CD3^+CD8^+$ T cells increased in 21-35d, the content of IL-4 and IFN-γ decreased from day 7 to 21, IL-2 decreased from day 21 to 35; Compared with Group Ⅱ, the percentages of $CD3^+$, $CD3^+CD4^+$ T cells in Group Ⅲ and Group Ⅳ increased in 7-28d, $CD3^+CD8^+$ T cells in Group Ⅳ and Ⅴ decreased in 7-35d, the content of IL-2 in the Group Ⅳ to Ⅵ increased in 14-28d, IL-4 and IFN-γ in the Group Ⅳ to Ⅵ increased in 0-35d. The result showed that the high dose Compound Kuqin total polysaccharides can enhance the secretion of IL-2, IL-4, IFN-γ and increase the percentages of $CD3^+$, $CD3^+CD4^+$ T cells, and decrease the percentage of $CD3^+CD8^+$ T cells. Based on the results, we concluded that the total polysaccharides of compound Kuqin can regulate canine immune functions and improve the antiviral ability in parvovirus infection.

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INTRODUCTION

The puppy is susceptible to be infected by a highly contagious disease called canine parvovirus disease, clinically with severe vomiting, hemorrhagic enteritis, leukopenia and myocarditis as the main features, its infection rate can be as high as 100%, and its mortality rate ranges from 10% to 50%, sometimes up to 100% (Minakshi *et al.*, 2016). The disease has been universally considered as the second most dangerous infectious disease because of its short course, high incidence, strong infectivity and high mortality rate (Dunham and Daly, 2016; Miranda and Thompson, 2016; Luo *et al.*, 2017).

In recent years, researchers had carried out a series of studies on the prevention and treatment of canine parvovirus and obtained some achievements, they developed the canine parvovirus nucleic acid vaccine, CPV recombinant live vector vaccine, canine parvovirus enteritis attenuated vaccine and canine parvovirus monoclonal antibodies (Miranda and Thompson, 2016), however, due to the reason that canine parvovirus 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, even leading to the poor clinical use of the vaccine (Ikeda *et al.*, 2000; Miranda and Thompson, 2016). However, researchers found that some polysaccharides of traditional Chinese medicine can effectively prevent and treat the disease, which provides a

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new research direction for controlling the canine parvovirus (Feng *et al.*, 2017; Yu *et al.*, 2017). The present study showed that T cell subsets play different roles in viral infections, different viruses can induce different T cell subsets in different immune response (Feeney *et al.*, 1995). The same viruses provoke different T cell subsets in different animals and at different infection stages. The role of different T cell subsets in anti-infection is closely related to the production of certain cytokines, and the differentiation of T cell subsets is regulated by different cytokines (Scott *et al.*, 1992). Enumeration of T cell subsets and testing cytokines are useful in the diagnosis of the severity of virus infection (Zaunders *et al.*, 1995; Wack *et al.*, 2011). Given this background, we can conclude that parvovirus can cause the T cell subsets and cytokines change in immune response, and the changes can reflect the physical condition of the disease. Our laboratory developed a new drug called Compound Kuqin that has a certain therapeutic effect for parvovirus disease, preliminary test results showed that the drug has a better prevention effect on the disease, and the total polysaccharides in the main composition of the compound has a strong inhibitory effect on the parvovirus disease (Lai *et al.*, 2014; Liu Fu *et al.*, 2016; Liu *et al.*, 2017). In the present study, we aimed to evaluate the preventing and protecting effect of Compound Kuqin total polysaccharides on canines infected by parvovirus through changes of blood T cell subsets and cytokines at different time. In addition, we tried to investigate the mechanism of Compound Kuqin total polysaccharides on reducing to the damage of canine parvovirus.

MATERIALS AND METHODS

Animals and treatment: One hundred and twenty 11-week-old Chinese Countryside Dogs (body weights 1.5±0.2 kg, half male and half female) were obtained from Experimental Animal Center of Southwestern University, Rongchang campus. All dogs were vaccinated with rabies vaccine and eliminated parasites by levamisole, and then they were isolated for observation for 1 week, to confirm the healthy and good nutritional status for the test. The animal experiments were performed according to the instructions (SYXK20110001) of the Medical Ethics Committee for the use of Experimental Animals at Southwest University.

Drugs: Compound Kuqin was prepared by the Traditional Chinese Medicine Laboratory in Rongchang campus of the Southwest University, and it was formulated by mixing eight herbals: *Scutellaria baicalensis Georgi*, *Radix sophorae Flavescentis* and *Radix pulsatillae*, etc. The polysaccharides of Kuqin was extracted by using the water decoction and ethanol precipitation method.

Determination of viral infectivity: An appropriate amount of CPV strain was inoculated into F81 cells which had been formed into monolayer. The cells were incubated for 90 min at 37°C in a 5% CO_2 atmosphere, then washed twice with PBS, replaced with maintenance fluid. We observed the cytopathic effect under the microscope every day, when more than 80% of the cells had cytopathic effect, the cells and culture solution freeze-thaw for 3 times, the supernatant was collected by centrifugation, then sub-packaged to 2 mL cryopreservation tubes, finally stored at -80°C until use. A portion of the strains were removed and the viral infective TCID50 was determined to be $10^{-4.76}$ / 0.1 mL using the Reed-Munch assay.

Model establishment: Every dog in the experimental group received the virus (5×10^5 PFU/mL) with perfusion 5 mL and subcutaneous injection 3 mL. Positive dogs in CPV test were used for further experiment.

Animal grouping and treatment: The dogs were randomly divided into 6 groups (n=20 /group): Group Ⅰ (the same volume of physiological saline was given twice a day), Group Ⅱ (only challenge without drugs), Group Ⅲ (Before challenge, oral administration of Astragalus polysaccharides twice a day for seven consecutive days), Group Ⅳ, Group Ⅴ, Group Ⅵ (Before challenge, oral administration of compound Kuqin total polysaccharides 2g/kg, 1g/kg, 0.5g/kg twice a day for seven consecutive days). We set the time point that 6 hours after the virus was inoculated as 0 d, and collect the blood sampling from the canine saphenous vein on days 0, 7, 14, 21, 28, and 35.

Detection of $CD3^+/CD4^+/CD8^+$ T lymphocytes in peripheral blood of dogs: The cell suspension was prepared according to the previously report (Kong *et al.*, 2007). Cell viability was detected by Trypan blue cell counting assay using a microscope, the living cell rate is above 90%. 1 mL cell suspension of each sample was added to the flow tube, 2500 rpm/min, centrifuge for 10 min, the supernatant was discarded. 100 μL PBS and 10 μL of CD3 - fluorescein isothiocyanate (FITC), CD4 - Phycoerythrin (PE), CD8 - *Allophycocyanin* (APC) antibody was added to the tube and incubated in the dark for 30 min, then 2 mL PBS was added, 2000 rpm/min, centrifuge for 5 min, and the supernatant was discarded, repeated the operation twice, finally, 200 μL PBS was added for mixing, and the T lymphocyte subsets were detected in the Third Military Medical University flow cytometry chamber. FACSCalibur (Becton Dickinson) was used for flow cytometry, and the data were analyzed by FlowJo software.

Detection the levels of IL-2, IL-4 and IFN-γ in serum of canine: The concentration of IL-2, IL-4 and IFN-γ in the canine serum were calculated by standard curve, and the absorbance (OD) was measured at 450 nm using a Microplate Reader according to the ELISA kit instructions.

Statistical analysis: All data were analyzed using a one-way ANOVA and the SPSS 20.0 statistical software. $P<0.05$ was considered as significant statistical difference.

RESULTS

Detection results of T lymphocyte subsets ($CD3^+/CD4^+/CD8^+$) in peripheral blood of dogs: Fig. 1 shows the gating strategy for flow cytometry assay, and Fig. 2-3 display the FACS graphs of $CD3^+/CD4^+/CD8^+$ T lymphocyte in dogs' lymphocyte liquid.

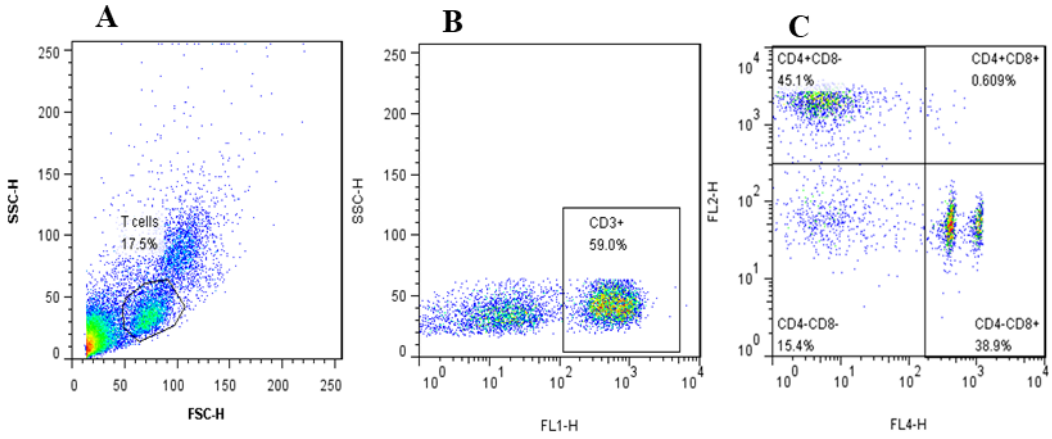

Fig. 1: Gating strategy for flow cytometry assay. First, T cells were identified (A), and gating on $CD3^+$ T cells was performed (B). Second, $CD4^+$ and $CD8^+$ T cells were gating on (C).

Compared with the Group Ⅰ, the percentage of $CD3^+$ T cells in canine peripheral blood lymph layer of Group Ⅱ significantly increased ($P<0.01$) at the day 7 and 14, but decreased significantly ($P<0.01$) at the day 28 and 35. Compared with the Group Ⅱ, the percentage of $CD3^+$ T cells in Group Ⅲ and Ⅳ increased significantly ($P<0.01$) at the day 28 (Fig. 4). Our result suggested that 2 g/kg Compound Kuqin polysaccharides increase the percentage of $CD3^+$ T cells in dogs infected with CPV.

Fig. 5 shows that the percentage of $CD3^+CD4^+$ T cells in Group Ⅱ decreased significantly ($P<0.01$) at the day 7, 14 and 35 when compared to the Group I. The percentage of $CD3^+CD4^+$ T cells in Group Ⅳ increased significantly ($P<0.01$) from day 21 to 35 when compared to the Group Ⅱ.Our data revealed that Compound Kuqin polysaccharides at the dose of 2 g/kg can up-regulate the percentage of $CD3^+CD4^+$ T cells in dogs infected with CPV.

As shown in Fig. 6, compared with the Group Ⅰ, the percentage of $CD3^+CD8^+$ T cells in Group Ⅱ increased significantly ($P<0.01$, $P<0.05$) at the day 7 and 21, but decreased significantly ($P<0.01$) at the day 14. Compared with the Group Ⅱ, the percentage of $CD3^+CD8^+$ T cells in Group Ⅲ, Ⅴ and Ⅵ decreased significantly ($P<0.05$, $P<0.01$) at the day 21. It suggested that Compound Kuqin polysaccharides at the dose of 1 and 2 g/kg can down-regulate the percentage of $CD3^+CD8^+$ T cells in dogs infected with CPV.

Detection results of the levels of IL-2, IL-4 and IFN-γ in canine serum: The results in Fig. 7 shows that the concentration of IL-2 in Group Ⅱ increased significantly ($P<0.01$) at the day 0, but decreased not significantly ($P>0.05$) from day 21 to 35 in comparison to Group Ⅰ. The content of IL-2 in Group Ⅲ, Ⅳ and Ⅴ significantly increased ($P<0.05$, $P<0.01$) at the day 21 by comparing with the Group Ⅱ.

In Fig. 8, compared with the Group Ⅰ, the content of IL-4 in Group Ⅱ decreased significantly ($P<0.01$) at the day 14 and 21; Comparing with the Group Ⅱ, the content of IL-4 in Group Ⅵ, Ⅴ and Ⅵ increased significantly ($P<0.01$) in day 0-35.

Changes of the IFN-γ level in dogs' serum of each group is shown in Fig. 9. When compared with the Group Ⅰ, the content of IFN-γ in Group Ⅱ increased significantly ($P<0.05$) at the day 0, but decreased significantly ($P<0.05$) in day 7-14. Compared with the Group Ⅱ, the level of IFN-γ in Group Ⅲ, Ⅳ, Ⅴ and Ⅵ increased significantly ($P<0.01$) in day 7-14.

The results indicated that three dose Compound Kuqin polysaccharides can enhance the IL-2, IL-4 and IFN-γ level in dogs infected with CPV.

DISCUSSION

Canine parvovirus disease characterized by two typical clinical symptoms, the dogs of all ages have enteritis with vomiting and diarrhea, and the puppy less than 3-months-old has the myocarditis and subsequent heart failure (Nandi and Kumar, 2010; Gu *et al.*, 2015). From some similar researches about virus infection, we find that T lymphocyte subsets and cytokines change with the progression of Canine parvovirus disease (Zaunders *et al.*, 1995; Wack *et al.*, 2011). In our study, compared with Group Ⅰ, the percentage of $CD3^+CD4^+$ T cells in Group Ⅱ decreased in 0-35d, the percentage of $CD3^+$ T cells decreased and $CD3^+CD8^+$T cells increased in 21-35d, the content of IL-4 and IFN-γ decreased from 7 to 21 days, IL-2 decreased from 21 to 35 days, suggesting that the virus invaded the body and triggered the immune response , the changes of T cell subsets and cytokines occurred, thus resulting in the destruction of the virus (Ng *et al.*, 2013; Wherry and Kurachi, 2015).

T cell subsets and their subgroups are important immune cell groups in animals, which play an important role in cellular immune function. CD3, CD4 and CD8 are important markers of T cell surface. CD3 is only present on the surface of T cell surface, it is necessary for TCR expression and signal transduction. Its main function is to transfer the antigen information of TCR to the cell, then the activation process in cells will be initiated, so it works as the activation of T cells in the early stage after antigen stimulation (Yang *et al.*, 2003). CD4 and CD8 were expressed on the surface of T lymphocytes in different functional subpopulations, namely $CD4^+$ and $CD8^+$ T cell subsets. $CD4^+$ has helper T cell (Th) function, $CD8^+$ has inhibitory T cells (Ts) and cytotoxic T cell function (Tc/CTL). In the immune response, the interaction in vivo, mutual antagonism between $CD4^+$ T cells and $CD8^+$

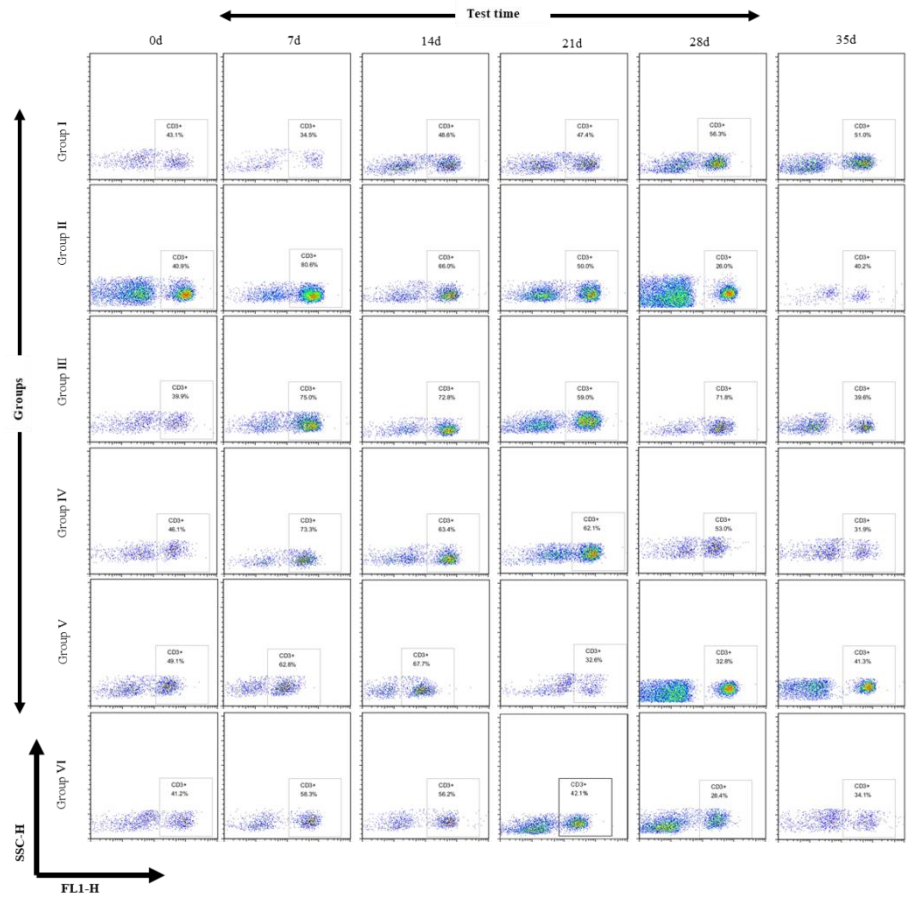

Fig. 2: The FACS graphs of $CD3^{+}$T lymphocyte in dogs' lymphocyte liquid.

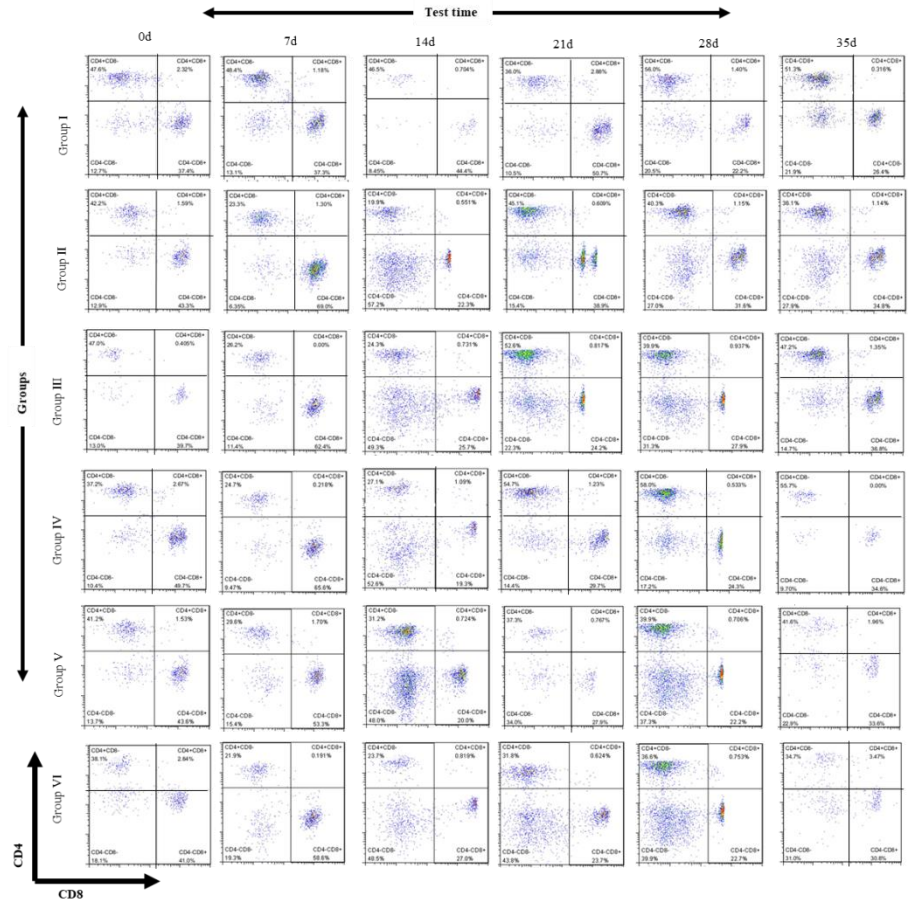

Fig. 3: The FACS graphs of CD4/CD8 T lymphocyte in dogs' lymphocyte liquid.

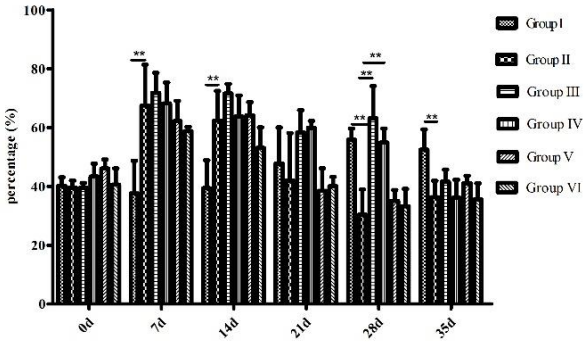

Fig. 4: Changes of the $CD3^+$T lymphocyte in dogs' lymphocyte liquid. * *P*<0.05. ** P<0.01. The same as below.

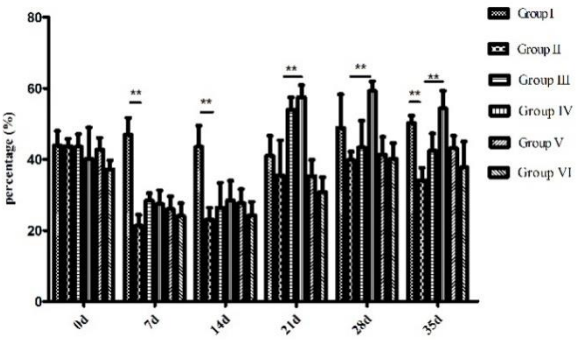

Fig. 5: Changes of the $CD3^+CD4^+$T lymphocyte in dogs' lymphocyte liquid.

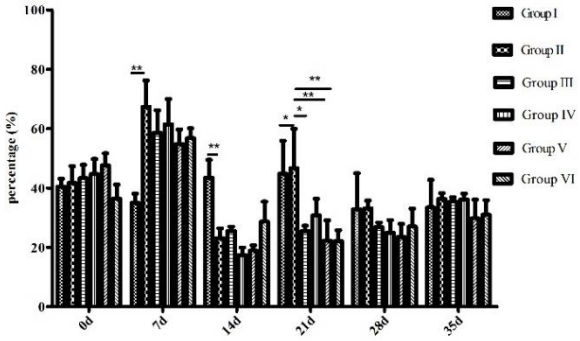

Fig. 6: Changes of the $CD3^+CD8^+$T lymphocyte in dogs' lymphocyte liquid.

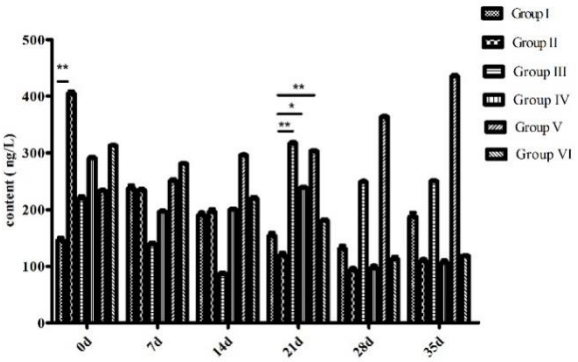

Fig. 7: Changes of the IL-2 content in dogs' serum.

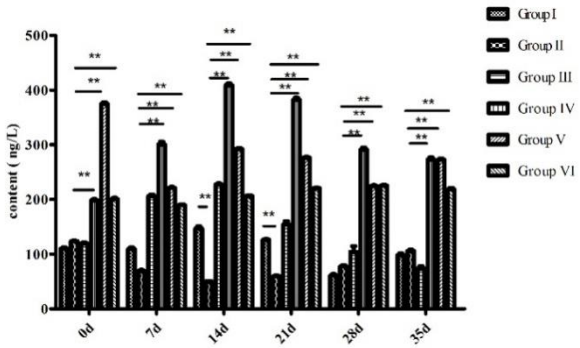

Fig. 8: Changes of the IL-4 content in dogs' serum.

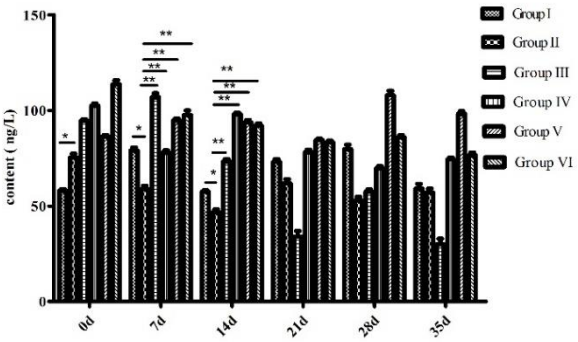

Fig. 9: Changes of the IFN-γ content in dogs' serum.

T cells maintain the balance of the body's immune system (Yang *et al.*, 2003). It has been reported that HIV (Human Immunodeficiency Virus) infection can induce the changes of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and the ratio of $CD4^+/CD8^+$ can reveal the severity of virus infection (Zaunders *et al.*, 1995) the percentage of $CD4^+$ increased and the $CD8^+$ decreased, the ratio of CD4/CD8 is improved, it implies the enhancement of immune function(Yan *et al.*, 2014). In this paper, compared with Group Ⅱ, the percentages of $CD3^+$, $CD3^+CD4^+$ T cells in Group Ⅲ and Group Ⅳ increased in 7-28d, $CD3^+CD8^+$ T cells in Group Ⅳ and Ⅴ decreased in 7-35d. The results indicate that the high and middle dose of compound Kuqin total polysaccharides can regulate the changes of T lymphocyte subpopulations and control the destructive effect of virus when the virus invades the body, and play the vital role of protecting the body. It is similar to the existing research findings (An *et al.*, 2014; Liu *et al.*, 2017).

Cytokines are a kind of small proteins that are important in modulating the balance between humoral and cell-based immune responses. IL-2 is mainly secreted by Th1 cells, Tc cells, NK cells, and its main biological effects are promoting T lymphocyte proliferation, differentiation and cytokine production, enhancing the activity of Tc cells, NK cells and LAK cells, promoting B cell proliferation and antibody production (Yang *et al.*, 2003). The biological effects of IL-4 mainly secreted by Th2 cells and mast cells are promoting B lymphocyte proliferation, IgE expression and mast cell proliferation, inhibiting Th1 cells, enhancing macrophage and Tc cell function. IFN-γ mainly secreted by Th1 and NK cells, the main biological effects are promoting the macrophage activation, the presentation of antigens, interfering with viral replication, anti-tumor, anti-parasite (Yang *et al.*, 2003). Endogenous IL-2 is a cytokine with a wide range of biological activity, mainly caused by pathogenic stimuli and T cell ($CD4^+$ Th1 and

$CD8^+$) generated. IL-2 can induce the proliferation and production of toxic T cells and directly kill the virus in the early stages of the pathogen into the body (Taniguchi and Minami, 1993). IFN-γ is secreted by primary infected cells when infected by virus at the first time, although it cannot inhibit viral replication in infected cells, it can be released into the surrounding cells, stimulating these cells to produce antiviral proteins and preventing the virus from proliferating. The reduction of IFN-γ can block the above process, while the increase in IL-4 will enhance the stimulation on B cells and mast cells, but also induced B cells to produce high levels of IgE and IgG1. IL-4, which was considered to be the macrophage activating factor, can enhance the antigen presentation ability of the cloned macrophage cell line, the realization of this function is due to the increased expression of antigen (Liu and Yue, 2008; Wan and Kang, 2010). In previous studies, Baicalin joint resveratrol retention enema can increase IL-2, IFN-γ levels of RSV infection rats (Cheng *et al.*, 2014), and the Compound Kuqin can promote the secretion of IL-2, IL-4 and IFN-γ (Liu *et al.*, 2017). Our study shows that the content of IL-2 in the Group Ⅳ to Ⅵ increased from day 14 to day 28 by comparing to the Group Ⅱ, IL-4 and IFN-γ in the Group Ⅳ to Ⅵ increased from day 0 to 35, suggesting that the total polysaccharides of Compound Kuqin can promote the secretion of IL-2, IL-4 and IFN-γ in canine. The results indicate that the Compound Kuqin total polysaccharides can induce the changes of the cytokines, control the destruction of the virus, and thus play a role in protecting the body.

Conclusions: In conclusion, Compound Kuqin total polysaccharides can regulate the $CD3^+$, $CD4^+$, $CD8^+$T lymphocytes and the secretion of corresponding cytokines in canines' body, and play a role of protecting the body.

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Authors contribution: JL conceived and designed the research. QYW and HQ did the experiment and analyzed the data. JL, QYW and HQ wrote this paper, JL finally approved the version.

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