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INFLUENCE OF ZHUQIN FORMULA EXTRACT ON THE CELLULAR AND HUMORAL IMMUNE RESPONSE IN CYCLOPHOSPHAMIDE-IMMUNOCOMPROMISED MICE

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ABSTRACT

The aim of this study was to evaluate efficacy of extract from traditional Chinese medicine Zhuqin formula (ZQE) in cyclophosphamide-immunosuppressed mice. A total of 340 four weeks old Kunming mice were taken for the study. The study is divided into two parts. One part is to determine a safe dose of ZQE by gastric perfusion in mice with an acute toxicity and a maximum tolerance test, and the second part of the presented study indicates the immunorestorative effects of ZQE dependent of the doses applied in mice treated with cyclophosphamide, including the carbon clearance index, the weight ratio of immune organ, serum hemolysin, T-, B-lymphocyte transformation rate and IL-2, IFN-γ, and IL-4 secretion. The acute toxicity test indicated that LD_{50} of ZQE by gastric perfusion in mice was > 5,000 mg/kg.Themaximum tolerated dose of ZQE (oral administration) was > 120 g/kg. In the treating immunosuppressed mice test, three doses of ZQE groups could increase the carbon clearance index, the weight ratios of spleen and thymus, serum hemolysin level, promote the secretion of IL-2, IL-4, and IFN-γ, and T-, B-lymphocyte transformation rate by 1.81-, 1.73-, 2.67-, 2.36-, 1.96-, and 2.69-fold of model group,respectively, and a 30mg/kg dose of ZQE presented a significantly better outcome, compared with the 40 and 20 mg/kg doses.

Keywords: cyclophosphamide; immunosuppression; spleen; thymus; cytokine; mice.

INTRODUCTION

In recent years, theoretical and practical researches have indicated that adoption of immune modulators for the improvement of the non-specific immune activity can enhance the humoral and cellular immune responses to vaccines; these methods will be able to improve vigor and disease resistance (Watanabe *et al*. 2010). The five major forms of therapy for the treatment of immunosuppression are as follows: immune stimulating complexes, liposome, cell-based therapies, immune stimulation by DNA, and natural products (House and Selgrade, 2010; Holsapple and O'Lone, 2012). The first four immune-modulators have limited effect on boosting the immune response, and also present adverse reactions, such as tigecycline-related digestive system adverse events (Khawcharoenporn and Apisarnthanarak, 2014).

In addition, issues such as, the drug resistance and recombinant interferon cost are common among them. Traditional Chinese medicine are natural products that have bi-directional effects on regulating immune function; these effects can be influenced by many factors such as the dose, physical status, drug compositions, and compatibility of medicines (Daligand *et al*. 2005; Chamilos and Kontoyiannis, 2006). Zhuqin formula (ZQ) is one such traditional Chinese medicine which is prepared from *Atractylodes*, *Poria*, *Radix Astragali*, and *Radix Scutellariae*. The first three herbs have effects on invigorating the spleen for dieresis, and the fourth herb has effects on eliminating dampness, strengthening body resistance by eliminating diseases, and invigorating the spleen for dieresis, and it also presents antidiarrheal properties. The main biological activity of Zhuqin formula is baicalin, which is a flavonoid glycoside obtained from the dried roots of *ScutellariabaicalensisGeorgi*. Baicalin has displayed a wide range of pharmacologic effects, including heat-clearance, increasing urination and protection of the liver. In general, it has been used to treat oxidative stress, improve body's immune and decrease H_2O_2-induced cytotoxicity (Lin *et al*. 2014; Zheng *et al*. 2014). Since this medicine has substantial benefits, it is used for the treatment of immunosuppression diseases, which are usually characterized by lassitude, lethargy, dry skin, anal relaxation, pale yellowish viscous stool commonly with undigested food, bulging belly, rapid weight loss, and a sunken appearance to the eye socket (Cho *et al*. 2013; Peng *et al*. 2014).

Studies have shown that immunosuppression patients are often characterized with: a reduction in thymus and spleen weight as well as ultrastructural changes of these immune organs; decrease in NK cell activity, ADCC function, cytokines secretion, and macrophage phagocytosis, tumor killing, NO-producing activities; low levels of serum immune globulins; changes in T cell subsets; impaired immune function of

red blood cells; decreased levels of immune factors (Wang*et al*. 2008). Cyclophosphamide is a cytotoxic drug that can inhibit mitosis due toits strong immunosuppressive effects. So there are many studies about model of cyclophosphamide-immunocompromised micein some immunology researches (Yoon *et al*. 2009; Huang *et al*. 2013).It showed that in spleen lymphocytes transformation test, the NK cell activity of mice with cyclophosphamide reduced (Huang *et al*. 2013). Gao Peng studied that white blood cells and macrophages numbers of C57BL/6J mice with intraperitoneal injection of 20 mg/kg reduced (Peng *et al*. 2004). It showed that these immunosuppression animals are generally characterized with the decline of the weight ratio of thymus and spleen, NK cell activity and cytokines secretion (Wang *et al*. 2008).

The purpose of this study is to evaluate efficacy of ZQE in cyclophosphamide-immunosuppressed mice, including the macrophage phagocytic rate, the weight ratio of immune organ, serum hemolysin, lymphocyte transformation rate, and levels of serum IL-2, IFN-γ, and IL-4.

MATERIALS AND METHODS

Preparation of Zhuqin Extract (ZQE): The natural herbs, *Atractylodes*, *Radix Scutellariae*, the root of Chinese *Pulsatilla*, *FructusGardenia*etc, were purchased from Chongqing RongchangTong JunGe drugstore. They were identified by Juan Liu, a professor of pharmacy at the Southwest University, China. ZQE was composed of: *Atractylodes* (4 g); *Radix Scutellariae* (3 g); *PoriaCocos* (3 g); *Astragalus* (4.5 g); *roasted Radix Aucklandiae* (2.5 g); *PogostemonCablin* (2.5 g); *RhizomaZingiberis* (2 g); and *Licorice* (1.5 g) (Chinese patent application number 201310344433.4). ZQE was initially prepared with two extracts, liquid A and liquid B.Theprocessisng of liquid A was prepared as described: a prescribed amount of *Pogostemoncablin* and *Atractylodes* was mixed with 8-fold amount of distilled water and refluxed twice, the distillate was then recovered, and was labeled as 'liquid A'. Then liquid B was prepared as: a prescribed amount of *Radix scutellariae*, *PoriaCocos*, *Astragalus*, roasted *Radix aucklandiae*, *RhizomaZingiberis* and *Licorice* were mixed with 8-fold ethanol in volume fraction of 50% in an extracting tank, and were refluxed twice (2 hours each), then the distillate was recovered and filtered, and ethanol recovery was performed through an alcohol recovery tower, the remaining extract was labelled as 'liquid B'. Liquid B was then concentrated into clear paste. After cooling, liquid A and B were mixed together and this mixture is labelled as 'ZQE', which was then heated and concentrated into clear paste with a total concentration of 1 g/mL. A total of 23 g ZQE (Baicalin, net content of 8-9% byliquid chromatography, figure 1) was prepared, sterilized, and stored (Hong*et al*. 2015).

Animals: A total of 340 Kunming mice (170 male and 170 female), weighing 20±2 g, were bought from the Laboratory Animal Center of Chongqing Rongchang campus Southwest University, China. All the experimental protocols were approved by the experimental animal ethics committee of Rongchang campus of Southwest University. All mice were fed for a week under normal conditions (28±5°C, 40-50% humidity, water, feeding) before test.

Acute toxicity test: For acute toxicity test, a total of 50 Kunming mice were fasted (free water) for 12 h, and divided into five groups (10 mice each) using a randomized block design (Zhi and Song, 2010;The Chinese Ministry Of Agriculture Veterinary Drug Review Center, 2012). Mice in the Group 1-4 were administered with four doses of ZQE, which were diluted with saline according to 2:1 ratio: 100mg/mouse, 50mg/mouse, 25mg/mouse, 12.5mg/mouse, respectively, whereas, mice in the Group 5 were treated with same volume of physiological saline and were considered as blank control group. The behavioural and physiological activities and death of the mice were recorded (The Chinese Ministry Of Agriculture Veterinary Drug Review Center, 2012). This test was repeated once.

The maximum tolerance dose test(Zhi and Song, 2010): A total of 40 Kunming mice were fasted (free water) for 12 h, and were divided into two groups, experimental and blank control group (n = 20 each) using a randomized block design(Kundt and Glass, 2012; Toorawa *et al*. 2009). The experimental group mice were treated with ZQE (1 g/mL) at a dose of 0.4 mL/10 g weight, which was repeated twice at 4 h intervals. Mice in blank control group were treated with 0.4 mL/10 gsaline, which was repeated twice at 4 h intervals. Then the parameters such as behavioural state, urine and feces, death etc. in the mice groups were observed for one week and recorded. The mice were weighed after one week, and blood samples were obtained for detecting the biochemical indexes [including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), glucose (Glu), total bilirubin (T-Bil), total protein (TP), serum albumin (ALB)] using a blood biochemical analyzer SBA-Diagnosis (Shanghai Shuangbo Biological Technology Co., Ltd.), and blood cell counts (including hemoglobin, red blood cells, white blood cell count, lymphocyte count, platelet count, neutrophil count) using a blood cell analyzer PE-6300VET (Shenzhen Pukang Electronics Co., Ltd.). Finally, the mice were dissected to observe the change in appearance of heart, liver, spleen, lung and kidney.

Mice grouping in the treating immunosuppressed mice test: A total of 200 Kunming mice weighing 20±2 g, were randomly divided into 5 groups (n = 40 each, male and female in equal), including blank group, model group,

and ZQE groups at three dose. Blank control group mice were treated with distilled water (30 mL/kg); model group mice weretreated with distilled water (30 mL/kg) seven-times per a week, was administered prior to intraperitoneal injection of cyclophosphamide (4 mg/mouse; Shanxi Pude Pharmaceutical Co., Ltd., 20120304) (Okawa and Yamada, 2002). In the three dose groups, ZQE was diluted with distilled water to high dose (2 mg/mL), medium dose (1.5 mg/mL), and low dose (1 mg/mL), and was administered once daily with the dose of 0.8 mg/mouse, 0.6 mg/mouse, 0.4 mg/mouse respectively. Followed by administrating the doses seven-times, cyclophosphamide (4 mg/mouse) was injected intraperitoneally. After 24 h, the mice were fasted for 4 h (free water) and were used for the following assays:

(i) Determination of the carbon clearance index

The carbon clearance index was determined according to the previous literature (Kolm *et al*. 2005). In brief, eight mice from each group were given tail-vein injections of India ink (0.1 mL/10g), and blood samples (20 µL) were taken from the orbital sinus at 2 min and 10 min after injection, respectively. After that 2 mL of 0.1% Na_2CO_3 was added immediately and OD_{650}nm was measured and the carbon clearance index (K) and the corrected clearance index (α) were calculated by the following formula:

$K= (lgA1-lgA2)/(T2-T1)$,

$\alpha = K1/3 \bullet$body weight/(liver weight + spleen weight)

Where: A1, A2 represent the OD_{650nm} value from the two blood samples, respectively; T2 and T1 represent the time when taking the two blood samples.

(ii) Determination of immune organ index

Eight mice from each group were killed, and the spleen and thymus were taken and weighed. The weight ratios of spleen and thymus were calculated by the following formula:

The weight ratio of spleen = spleenweight mg / mouse body weight (g);

The weight ratio of thymus = thymus weight mg / mouse body weight (g).

(iii) Determination of the serum hemolysin level

The serum hemolysin levels were determined according to the previous literature (Bliznakov, 1976). In brief, eight mice from each group were injected intraperitoneally with 0.2 mL of 5% chicken red blood cell (CRBC, $1\text{-}2\times10^6$ cells/mL, obtained from local cock of Chongqing Rongchang, China), seven-times per a week. After 24 h of the last injection, blood samples were taken from the orbital sinus and the serum was separated by centrifuging the sample for 10 min at 2,000 rpm. Serum samples were diluted at a ratio of 1:100 in physiological saline. The diluted serum (1 mL), 5% CRBC suspension (0.5 mL), and 10% guinea pig serum (0.5 mL) were mixed and incubated at 37°C for 30 min. Then the reaction was terminated by chilling to 0°C. The supernatant was separated by centrifugation for 10 min at 2000 rpm and the absorbance value (A_{540nm}) was measured using a UV spectrophotometer. The hemolysin value was represented as half value of hemolysis (HC_{50}).

(iv) Determination of T- and B-lymphocyte transformation rate

T- and B-lymphocyte transformation rate was determined according to the previous literatures (Dessau*et al*. 2014; von Baehr, 2014). In brief, blood samples from the orbital sinus of eight mice from each group were taken, and lymphocytes were prepared at a concentration of 1×10^6 /mL for further use. Lymphocyte suspension (200 µL) was added into each well of a 96-well plate, followed by addition of 15 µL concanavalin A into each well (6 mg/L final concentration). Eight repeated tests were performed for each sample and after 72 h of incubation at 37°C in a 5% CO_2 incubator, MTT assays were performed as follows: 100 µL supernatant was removed 4 h before test and 20 µL MTT detecting solution was added; after 4 h of further incubation, the supernatant was removed and 150 µL DMSO was added to terminate the reaction, the sample was then mixed by vibrating it horizontally and the OD_{570nm} value was measured using an ELISA plate reader to obtain the T-lymphocyte transformation index. The calculation of B-lymphocyte transformation index was identical except that concanavalin A was replaced by lipopolysaccharide.

(v) Determination of serum IL-2, IFN-γ, and IL-4 levels

Blood samples from the orbital sinus of eight mice from each group were taken and serum samples were prepared. The levels of IL-2, IFN-γ, and IL-4 were measured using ELISA kits according to the manufacturer's instructions.

Statistical analysis: Data were presented as the mean ±SE and analyzed using the SPSS® statistical package, version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Differences between the values were determined using a Student's t-test. P-value<0.05 was considered to be statistically significant, and P-value<0.01 was considered to be statistically significant.

RESULTS

Acute toxicity test: In the repeated acute toxicity tests, no mouse was found died, which indicated that the LD_{50} of ZQE was greater than 5,000 mg/kg.

The maximum tolerance dose test: Results of the maximum tolerance dose test showed that, the mice group administered with ZQE (120 g/kg) exhibited normal behaviours, their weight decreased slightly after the administration, and there were no deaths during the study, however no significant difference was found when these outcomes were compared with blank control group (Table 1). When dissected, the main organs (heart, liver, spleen, lung and kidney) showed no evident change in appearance. Therefore, the maximum tolerance dose of

ZQE (oral administration) to mice was more than 120 g/kg.

When compared to blank control group, the platelet count was significantly increased after one week administration of 1 g/mL ZQE ($P< 0.01$). The white blood cell count and lymphocyte count showed significant difference between the experimental group and blank control group ($P< 0.05$). Table 2 shows that all the blood cell indexes were in normal range, and in the Table 3, it is evident that the AST and ALT levels were significantly decreased compared with blank control group ($P< 0.05$ and $P< 0.01$, respectively). Compared with the blank control group, TP and T-Bil levels were significantly increased ($P< 0.05$), and all the biochemical indexes were in normal range, indicating that ZQE had little effect on them.

The treating immunosuppressed mice test

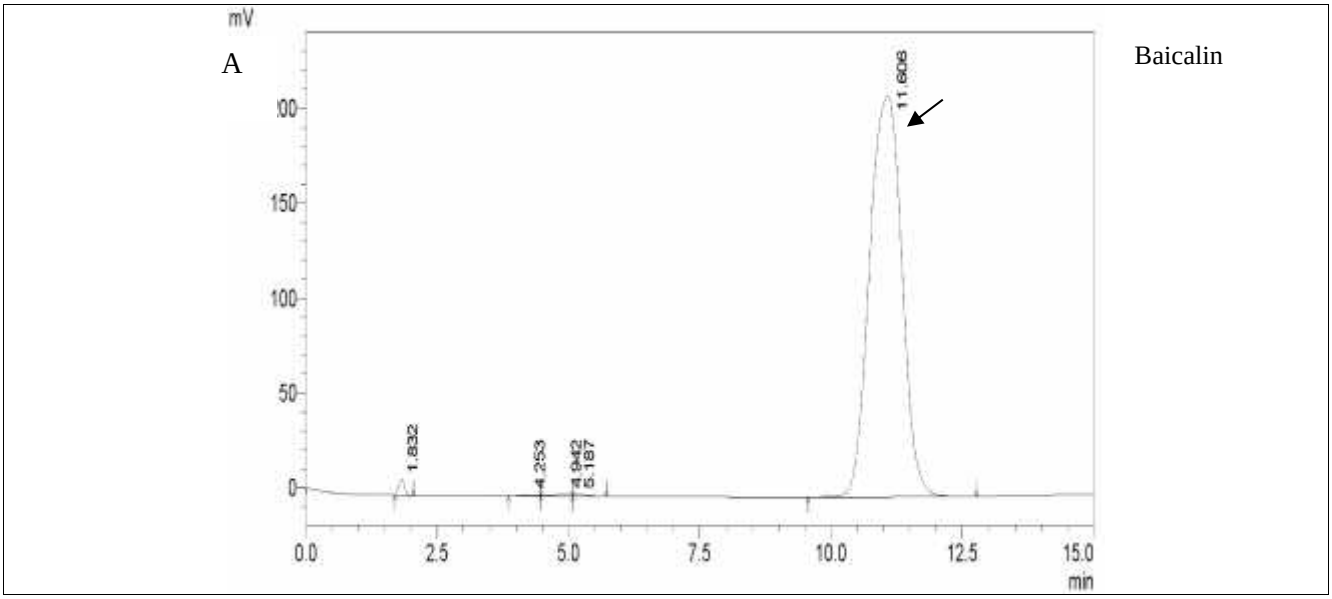
The carbon clearance index: As shown in Table 4, when compared with the blank control group, the carbon clearance index of model group was significantly decreased ($P<0.01$). Three ZQE groups increased significantly compared between and model group ($P<0.01$). And the 40 mg/kg dose group and 30 mg/kg dose group presented more satisfactory results than20 mg/kg dose group ($P< 0.05$).

The weight ratio of immune organ: As shown in Table 5, the immune organ indexes of model group were significantly decreased compared with that of blank control group ($P< 0.01$), and the spleen indexes of ZQE groups were evidently increased. The 40 mg/kg dose group showed significantly satisfactory results than that of 30 mg/kg dose group ($P< 0.05$) and 20 mg/kg dose group ($P< 0.01$), and a certain dose-effect relationship existed among these groups.

The serum hemolysin level: As shown in Table 6, when compared with blank control group, the hemolysin level (HC_{50}) was significantly decreased in model group ($P< 0.01$). It was observed that after the administration of ZQE, HC_{50} was significantly increased compared with model group ($P< 0.01$); the 30 mg/kg dose group showed significantly higher level of HC50 than the 40 mg/kg dose group ($P< 0.05$) and 20 mg/kg group ($P< 0.01$).

Detection of T- and B-lymphocyte transformation rate: As shown in Table 7, compared with blank control group ($P< 0.01$), the T- and B-lymphocyte transformation in model group was significantly decreased; compared with 20 mg/kg ZQE ($P< 0.01$), the administration of 40 mg/kg ZQE and 30 mg/kg ZQE significantly increased the T-lymphocyte transformation rate; and finally compared with model group ($P< 0.01$), the B-lymphocyte transformation rates of ZQE groups were significantly increased.

Measurement of serum IL-2, IL-4, and IFN-γ: As shown in Table 8, levels of serum IL-2, IL-4, and IFN-γ were significantly decreased in model group, when compared with blank control group ($P< 0.01$). However, after the administration of ZQE, levels of serum IL-2, IL-4, and IFN-γ were significantly increased, when compared with model group ($P< 0.01$). The 40 mg/kg dose group showed a better outcome than 30 mg/kg dose group and 20 mg/kg dose group.



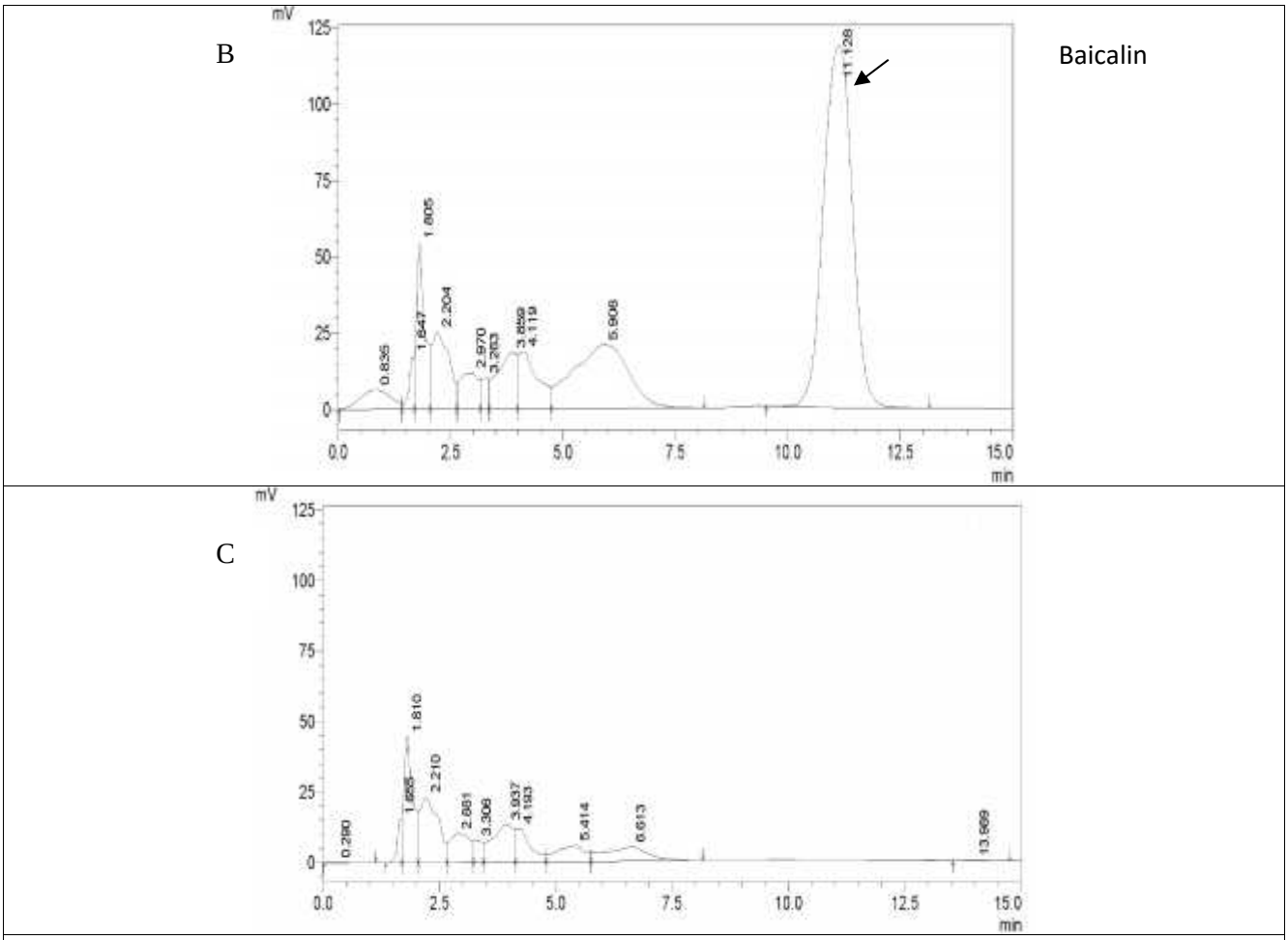


Fig. 1. The concentration of baicalin was determined by HPLC. Panel A: baicalin control. Panel B: sample. Panel C: negative. Chromatography was performed on a Wonda Sil® C18 column(4.6 mm×250 mm, 5 ìm), and acetonitrile-methanol-0.2 % phosphoric acid solution (V:V =52:48) as the moblie phase of baicalin was used on the wavelength of 274 nm with 10 ìL of the injection volume, at the flow rate of 1.0mL/mL and column temperature of 30 ℃.

Table 1. Weight of mice before and after the maximum tolerance dose test (n = 20)

Groups	Before test (g)	After test (g)
ZQE group	20.91 ± 1.31	20.02 ± 1.94
Blank control group	20.36 ± 1.64	21.06 ± 1.46

Table 2. Blood cell counts one week after the administration of ZQE (n = 20)

Indexes \ Groups	Blank control group	ZQE group
Hemoglobin (Hb, g/L)	120 ± 6.75	134 ± 9.42
Red blood cells (Ec, $\times10^{12}$/L)	3.9 ± 0.27	3.4 ± 0.41
White blood cells (Lc, $\times10^{9}$/L)	6.8 ± 0.92	10.5 ± 0.73 *
Lymphocytes (TLC, $\times10^{9}$/L)	3.7 ± 0.58	2.9 ± 0.71*
Platelets (PLT, $\times10^{9}$/L)	246 ± 17.21	314 ± 12.41**
Neutrophils (ANC, $\times10^{9}$/L)	4.5 ± 0.71	6.8 ± 0.91 **

*P< 0.05; **P< 0.01 *vs* blank control group, P> 0.05 (not marked).

Table 3. Blood biochemical indexes one week after the administration of ZQE (n = 20)

Indexes \ Groups	Blank control group	ZQE group
AST (U/L)	73.31 ± 3.08	62.77 ± 2.41*
ALT (U/L)	68.13 ± 2.01	43.16 ± 1.91**
Glu (mmol/L)	7.28 ± 0.92	8.07 ± 1.06
Cr (μmol/L)	66.26 ± 2.21	61.79 ± 2.89
TP (g/L)	67.58 ± 3.76	79.86 ± 4.68*
ALB (g/L)	41.56±0.77	42.09±0.89
T-Bil (μmol/L)	24.73 ± 0.52	31.92 ± 1.20 *

*P< 0.05; **P< 0.01 *vs* blank control group.

Table 4. The influence of ZQE on the carbon clearance index in cyclophosphamide-compromised mice (n = 8)

Groups	ZQE (mg/mouse)	Corrected clearance index
Blank control group	-	6.017 ± 0.12bb
Model group	-	3.078 ± 0.19aa
ZQE groups	0.8	5.501 ± 0.11abd
	0.6	5.564 ± 0.01abbcd
	0.4	5.230 ± 0.14abbd

P values: aP< 0.05; aa$P$< 0.01 *vs* blank control group;bP< 0.05, bb$P$< 0.01 *vs* model group; cP< 0.05,cc$P$< 0.01 *vs* 20 mg/kg dose group; dP< 0.05, dd$P$< 0.01 *vs* 30 mg/kg dose group; eP< 0.05, ee$P$< 0.01 *vs* 40mg/kg dose group. The same is as below.

Table 5. The effects of ZQE on the weight ratio (index) of thymus and spleen in mice treated with cyclophosphamide (n = 8)

Groups	ZQE (mg/mouse)	Spleen index (mg/g)	Thymus index (mg/g)
Blank control group	-	4.29 ± 0.18 bb	1.67 ± 0.09 bb
Model group	-	2.13 ± 0.12aa	0.60 ± 0.17aa
ZQE groups	0.8	3.97 ± 0.11abccdd	1.56 ± 0.09bbc
	0.6	3.69 ± 0.16aabbcee	1.60 ± 0.12bbc
	0.4	3.15 ± 0.21aabbdee	1.20 ± 0.08abbde

Table 6. The influence of one week ZQE administration on the serum hemolysin level in cyclophosphamide-immunocompromised mice (n = 8)

Groups	ZQE (mg/mouse)	HC_{50}
Blank control group	-	1.606±0.18 bb
Model group	-	0.672±0.20aa
ZQE groups	0.8	1.571±0.16abbccd
	0.6	1.589±0.21bbcd
	0.4	1.375±0.27aabbcee

Table 7. The influence of ZQE on T- and B-lymphocyte proliferation in cyclophosphamide-immunocompromised mice (n = 8)

Groups	ZQE (mg/mouse)	T-lymphocyte (OD_{570nm})	B-lymphocyte (OD_{570nm})
Blank control group	-	0.460 ± 0.17 bb	0.420 ± 0.16 bb
Model group	-	0.210 ± 0.09 aa	0.155 ± 0.12aa
ZQE groups	0.8	0.409 ± 0.12abbccd	0.417 ± 0.06 bb
	0.6	0.412 ±0.08abbcc	0.405 ± 0.14 bb
	0.4	0.301 ± 0.10aabbddee	0.403 ± 0.16 bb

Table 8, The influence of ZQE on serum IL-2, IL-4, and IFN-γ in cyclophosphamide-immunocompromised mice (n = 8)

Groups	ZQE (mg/mouse)	IL-2 (ng/L)	IL-4 (pg/mL)	IFN-γ (ng/L)
Blank control group	-	25.093 ± 0.362^{bb}	24.793 ± 0.113^{bb}	437.014 ± 2.413^{bb}
Model group	-	19.828 ± 0.179^{aa}	18.929 ± 0.267^{aa}	240.000 ± 2.920^{aa}
ZQE groups	0.8	24.756 ± 0.299^{bbccd}	24.022 ± 0.373^{abbcc}	417.264 ± 3.131^{bbcd}
	0.6	23.653 ± 0.326^{bbcc}	23.723 ± 0.428^{abbcc}	395.588 ± 2.744^{abbe}
	0.4	$21.765 \pm 0.163^{bbddee}$	$22.952 \pm 0.428^{aabbddee}$	400.886 ± 2.372^{abbe}

DISCUSSION

ZQE formula is prepared to treat spleen deficiency diarrhea syndrome. It is effective in eliminating dampness, strengthening body resistance and eliminating diseases, invigorating spleen for dieresis and it also presents antidiarrheal properties. Recent researches have indicated that the main field for immunological studies in spleen deficiency syndrome is immune function change. These current research concepts have certain common points with the traditional Chinese medicine theory that a healthy spleen can keep one from evils (diseases) of the four seasons and internal impairment of the spleen and stomach causes various diseases (Shu and Nan, 2008).

The complex network of immune organs, tissues, immune cells and cytokines, and other components of the immune system acts synergistically through circulating lymphocytes and immune molecules, thus playing important roles such as anti-infection and anti-tumor (Dodds, 2002). Certain drugs, such as exogenous chemicals could lead to reduced immune function by directly damaging the immune system, and making the body vulnerable to invasion by external virus and bacteria. Cyclophosphamide [chemical name: N, N-bis (2-chloroethyl) tetrahydro-2H-l,3,2-oxaphosphorin-2-amine,2-oxide monohydrate] plays anti-tumor roles by mainly producing glyciphosphoramide in liver, which deactivates biological macromolecules such as DNA by interacting with them (Isono *et al*. 2014; Jamieson*et al*. 2014; Meng *et al*. 2014; Roy *et al*. 2014). Since, the intraperitoneal injection of cyclophosphamide may damage the structure and function of lymphocytes DNA and inhibit the DNA replication, the proliferation of lymphocytes was impaired and antibody production was inhibited (Alves *et al*. 2014). In this study, it was found that the weight ratios of spleen and thymus were reduced after cyclophosphamide injection, which indicated a reduction of immunity; these findings were in accordance with previous reports of Lis *et al*. (Okawa and Yamada, 2002; Lis *et al*. 2013).

According to the provisions for drug therapy, clinical use of drugs must be in safe dose range. If any drug dose is above a certain dose level, it will cause damage, and if these levels are much higher than the recommended dose, it will cause loss of function or impairment of the organs, and even death (Bertram *et al*, 2001). In this study, as indicated by the maximum tolerance test, no experimental mice died and their behavioral state and the blood cell and biochemical indexes were in normal range. Furthermore, no abnormal change was observed in mouse organs, as shown by dissection. These results proved that ZQE formula is a safe medicine.

In this study, it was found that ZQE formula could increase the spleen index, thymus index, and serum hemolysin level. In addition, it could enhance the carbon clearance ability, T- and B-lymphocyte transformation, and promote the secretion of IL-2, IL-4, and IFN-γ to some extent. In animals, T-lymphocytes are mainly attributed to the cellular immunity, and B-lymphocytes can be transformed into plasma cells during antigen stimulation. A variety of specific antibodies was produced by plasma cells and was released into blood to prevent damage of corresponding antigens and foreign materials (Ingel *et al*. 2014; Yuan and Su, 2014). Lymphocyte proliferation is a critical procedure against foreign antigens in immune response, and this determines the number of effective lymphocytes required to remove foreign antigens and maintain homeostasis. Therefore, the lymphocyte number is highly related to the immune response intensity and immunological status of body (Ma *et al*. 2009). Baicalinandlicorice acid could enhance the proliferation and activity of helper T lymphocyte, promote IL-2, IFN-γ and IL-1 secretion, and inhibit IL-4, IL-10 and IL-8 secretion (He *et al*. 2011; Cheng *et al*. 2014). And they also inhibit the activation pathway of the complement system. Calcium as an important messenger molecule has an important role on the lymphocyte function, for instance, the regulation of lymphocyte division and the phagocytosis mediated by macrophages. The polysaccharide of ZQE could cause changes of calcium concentration within the cell and promote to bind to cell surface receptors of macrophages, to activate the receptor-dependent calcium channels with calcium influx and the activation of DNA polymerase and DNA synthesis in lymphocytes for promoting T cell proliferation and IL-2 production. ZQE also could affect some gene expressions of lymphocytes in the information

transfer process of lymphocytes. It indicated that different doses of ZQE had different effects on immunity of mice with cyclophosphamide, and these changes suggested that probiotics may affect body resistance and eliminate diseases by different mechanisms in mice.

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