

ADJUVANT EFFECTS OF SIJUNZI DECOCTION IN CHICKENS ORALLY VACCINATED WITH ATTENUATED NEWCASTLE-DISEASE VACCINE

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Abstract

Many Chinese Herbal medicines (CHMs) and their components have been reported to enhance immunity. In this study, the capacity for the Chinese herbal medicine, oral administration Sijunzi Decoction (SJZD) in stimulating Newcastle disease virus (NDV) immunity in chickens was examined. Serum was sampled on days 20, 30, 40, 50 and 60 and tissues were collected on days 20, 40 and 60, respectively. The immune responses were determined by means of hemagglutination inhibition test, immunohistochemistry examination and semi-quantitative RT-PCR. The results showed that SJZD could increase the antibody titers and the area coefficient of IgA secreting cells, promote the expression of IL-2 mRNA in the whole immune period and IFN- γ mRNA was increased in the initial stage. The SJZD used was safe with no adverse effects on chicken weight or survival, providing evidence for the use of SJZD as an oral adjuvant.

Keywords: Sijunzi Decoction; Attenuated Newcastle-disease vaccine; mucosal immunity adjuvant; Chicken

Introduction

Newcastle disease (ND) is a highly contagious and widespread disease which causes severe economic losses in domestic poultry, especially in chickens (Liu et al., 2003; Sinkovics and Horvath, 2000). Vaccination remains the most cost-effective biomedical approach for controlling the disease. Many researchers have found that oral immunization with attenuated Newcastle-disease vaccine could induce host immunity, and improve mucosal and systemic immune responses (Zuercher et al., 2006; Zhang et al., 2008; Zhang et al., 2007). However, oral vaccination needs more and repeated antigen doses to achieve the protective immune response level, because of degradation of the antigen by gastric acid and proteases present in the gastrointestinal tract (Hoshi et al., 1998; Holmgren et al., 1992, 2003). Therefore, it is necessary to develop strategies to induce mucosal immune responses effectively by using a small amount of antigen. An important factor which influences the effectiveness of mucosal immunization is the nature and efficacy of the adjuvant (Hoshi et al, 1998). A number of studies have demonstrated that the combinative application of vaccine with adjuvants or immunopotentiators could improve the efficacy of oral vaccination. Unfortunately, strong adjuvant activity is often correlated with undesirable side-effects, such as local stimulation, tissue damage and so on (Sun et al., 2009). Therefore, it is very important to develop immunopotentiators with high efficacy, low toxicity, and extensive availability.

Many Chinese herbal medicines (CHMs) and their components have been reported to enhance immunity. Combined use of vaccines with Chinese herbal medicines is emerging as one of the innovative approaches in adjuvant development. Particular advantages offered by herbal adjuvants in inducing cellular and humoral immunity are extremely safe, tolerable, ease to manufacture. Thus, the herbal adjuvants have enormous potentials for use in vaccinations against both pathogens and cancer (Petrovsky, 2006).

Sijunzi decoction has been used in China since Song dynasty (960-1279 AD) as a tonic. It ranks at the first top of

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tonifying formulas. The formula is traditionally believed to be immunoenhancer (Zhang Ed.,1990). There have been an increasing amount of papers on herbal immunopotentiators (Wang et al., 2006b). In the present study, the capacity for the Chinese herbal medicine sijnzi decoction (SJZD) in stimulating immunity against Newcastle disease virus (NDV) following oral administration to chickens was examined. We investigated the adjuvant effects of the Sijnzi Decoction (SJZD) with respect to mucosal and systemic immune responses. Additionally, The dose dependent responses of immune enhancement by SJZD were also evaluated.

Materials and Methods

Preparation of sijnzi decoction(SJZD)

The formula Sijnzi decoction consists of 4 crude herbs, i.e., Panax ginseng, Poria cocos, Atractylodes macrocephala and Glycyrrhiza uralensis at a ratio of 2:2:2:1. The herbs were purchased from a local Herbal Shop Tongrentang Herbal Company Ltd(Baoding, China) and authenticated by the Hebei provincial Bureau of herbal medicine. The herbs were boiled in water 10 times the weight of the herbs for 0.5 h and then the aqueous extract separated by filtration. The remaining herbs were decocted again for another 0.5 h as in the above. The two liquid parts were mixed and heated (50–60°C) through rotary evaporation to reduce the water content, and concentrated into 1:1 concentration decoction (Lenon et al.,2007), i.e., 1 ml of the concentrated extract was equivalent to 1g of the raw herb. Then, the herbal preparations were sterilized in sealed plastic bottles and diluted in distilled water to the low (0.125 g/ml), medium(0.25 g/ml), high(0.5 g/ml) dosages on the day of use.

Reagents

SP-9002 HistostainTM-Plus kits (Zhongshan Biotech Co., Ltd. Beijing, China) consists of Bloking solution, 3% H₂O₂, Biotin-goat anti-mouse IgG, Streptavidin peroxidase (S-A/HRP). Mouse anti-chicken IgA (Southern Biotech, Inc, USA), Diaminobenzidine (DAB, Sigma, USA). Moloney murine leukemia virus (M-MLV) reverse transcriptase, 5x RT buffer, Taq DNA polymerase and Oligo d(T)₁₈ primer, dNTPs, were products from Tiangen Biotech (Beijing, China).

Vaccine

The attenuated Newcastle-disease vaccine virus (La Sota strain, catalog no. 080519) was offered by Ruipu Biotech (Baoding, China) and diluted with sterilized phosphate-buffered saline (PBS, pH 7.4).

Animals

One-day-old white Leghorn chickens (male), purchased from Fanzhuang Poultry Farm, were housed in wire cages (60 cm×100 cm). Chickens were fed with the commercial starter diet, provided by the feed factory of Fangtian in Baoding city. Feed and water were supplied *ad libitum*. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the Agricultural University of Hebei.

Experimental design

One hundred and fifty chickens were randomly divided into five groups. Each chicken in herb groups A, B and C received different dosages of herbal treatments on day 12 as shown in Table 1 consecutively for 7 days. Group D was kept as non-herbal-non-vaccine control. Birds in Group E were non-herbal controls, receiving ND vaccine only. Birds in groups D and E were given equivalent amount of normal saline as in groups A, B, and C. On day 14, their average titer of maternal

antibody was 3.8 log₂ and the average bodyweight was 110±8g. Birds in groups A, B, C and E were orally vaccinated with 0.5ml (10⁶EID₅₀ per chicken) of attenuated Newcastle-disease vaccine virus, while group D received 0.5ml normal saline. The chickens of all the above-mentioned groups were vaccinated again on day 35 with the same dosages and in the same manners.

Sample collection

On Days 20 (D₂₀), 30 (D₃₀), 40 (D₄₀), 50 (D₅₀) and 60 (D₆₀), six chickens were sampled randomly from each group for determination of serum antibody. Blood samples (1.0 ml per chicken) were drawn into Eppendorf tubes from the main brachial vein of chickens and allowed to clot at 37 °C for 1 h prior to serum collection. Serum was separated by centrifugation and stored at -20 °C for use. On Days 20 (D₂₀), 40 (D₄₀), and 60 (D₆₀), tissue samples of five chickens were taken. The tissue samples of jejunum (three pieces of each tissue, 1 cm away from each other) were collected immediately, frozen in liquid nitrogen and then stored at -80 °C until RNA extraction and other analysis. Other tissue samples of duodenum and jejunum (at least three pieces, 1 cm away from each other) and Peyer's patch were fixed in Bouin's liquid.

Table 1: Animal treatment in different groups

Group	Exposure Group	N	Immunogen dose ml/chicken	Dose of SJZD (g/ml)
A	SJZD(L)+ND	30	0.5	0.125
B	SJZD(M)+ND	30	0.5	0.25
C	SJZD(H)+ND	30	0.5	0.5
D	Control	30	---	---
E	ND	30	0.5	---

Notes: SJZD(L), Low dose of SJZD; SJZD(M):Medium dose of SJZD; SJZD(H): High dose of SJZD; ND, Newcastle Disease vaccination. N, Number of chickens in each experimental group.

Table 2: Oligonucleotide primer sets used for semi-quantitative RT-PCR

Genes	Accession No.	Primer sets	Primer Sequences	Product Size (bp)
β-actin	L08165	F	CATCTATCGTGGGTCGC	547
		R	CTCCTTGATGTCACGCAC	
IL-2	AF017645	F	TATCGAAAAGAACCTCAAG	245
		R	CCAGAATGGACAGCAGA	
IFN-γ	NM_205149	F	GCTGACGGTGGACCTATT	240
		R	CAAGTCGTTTCATCGGGAG	

Measurement of ND Antibody

The antibody response to NDV-virus was determined by means of haemagglutination inhibition (HI). Briefly, two-fold serial dilution of serum, after inactivated at 56 °C for 30 min, were made in a 96-well, V-shaped bottom microtiter plate containing 50 µl of 0.9% NaCl in all wells and then 50µl of NDV antigen (four HA units) was added into all the wells except for the last row which served as the controls. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 10 min at 37 °C. Then 50 µl of a 1% rooster erythrocytes suspension was added to each well and re-incubated for 30min. A positive serum, a negative serum, erythrocytes, and antigens were also included as controls. The

highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal log₂ values of the highest dilution that displayed HI.

Immunohistochemical examination for IgA secreting cells

The fixed samples duodenum and jejunum were embedded in paraffin and serial sections of 5 µm were prepared. The sections were deparaphinized and hydrated, then the endogenous peroxidase activity was neutralized by 3% H₂O₂ for 30 min. The sections were treated with 5% normal goat serum in PBS for 30min to block non-specific binding and then stained with mouse anti-chicken IgA(1:200) at 4°C over night. The sections were rinsed three times with PBS for 5min each and then incubated with goat anti-mouse IgG at 37°C for 40 min. The sections were rinsed three times with PBS for 5min each, followed by incubation with S-A/HRP for 35 min at 37°C. After the sections were rinsed three times in PBS, the reactions were made visible with metal-enhanced diaminobenzidine (DAB).

Detection of Cytokine mRNA by Semi-Quantitative RT-PCR

The mRNA expression of the cytokines IL-2 and INF-γ were determined with a reverse transcriptional polymerase chain reaction (RT-PCR). The housekeeping gene, β-actin was used as an internal control. Total RNA was extracted from jejunum according to the RNA Simple Total RNA Kit's instruction and quantified by determining the optical density at 260 nm. RNA was reverse transcribed into cDNA with Oligo d(T)₁₈. The RT reaction mixture contained 20 µl: 1 µl of Oligo d(T)₁₈ primer, 2 µl of 2.5 mM stock of dNTPs and 11.5 µl of DNase-treated RNA in DEPC water were denatured at 70°C for 5 min and immediately chilled on ice for 3 min. The following reagents were added: 4 µl of 5x RT buffer, and 0.5 µl Ribonuclease inhibitor, 1 µl of 50 µunits /µl of TranScript RT. The contents were mixed gently and briefly centrifuged followed by incubation at 42°C for 50 min. Finally, the reaction was stopped by heating at 95 °C for 5min and all cDNA samples were stored at -80 °C prior to amplification. The resulting cDNA was subjected to polymerase chain reaction (PCR) with respective primers designed from the sequences of two cytokines. Amplification was carried out in a total volume of 25 µl containing 2 µl (10 µM) of each cytokine- specific primers (Table 2), 2.5 µl 10× PCR buffer, 2 µl dNTP, 2 µl cDNA and 0.5 µl Taq DNA polymerase. The PCR condition of IL-2 mRNA: at 94 °C for 4min (denaturation 94 °C for 50 s; annealing 54 °C for 50 s; polymerization 72 °C for 50 s), 31cycles, the reaction was extended at 72 °C for 10min; the PCR condition of IFN-γ mRNA: at 94 °C for 4min (denaturation 94 °C for 50 s; annealing 56 °C for 50 s; polymerization 72 °C for 50 s), 35 cycles, the reaction was extended at 72 °C for 10min. Different controls were set to monitor the possible contaminations of genomic and environment DNA both at the stage of RT and PCR. The PCR products were electrophoresed with 1% agarose gel and stained with ethidium bromide. The quantity of PCR products was analyzed by densitometer. The relative value of IL-2 or IFN-γ mRNA= (the value of IL-2 or IFN-γ mRNA)/(the value of β-actin), which can mean the high or low of expression of IL-2 or IFN-γ mRNA. All experiments were carried out in triplicate.

Safety tests

For assessment of potential adverse effects of SJZD, the weight of ten chickens each group was monitored on days 12 and 19. Any weight change in SJZD treated chickens was compared to group control to determine whether any deviation from normal growth rates occurred during the administration of SJZD. Additionally, Survival rates were also undertaken as an indicator of herbal toxicity relative to control group.

Statistical analysis

The areas of IgA secreting cells in 10 different microscope fields of well-oriented, intact intestinal villi in each

tissue (total 5×5 sections) was measured by Scion Image. The data were calculated by the area coefficient of IgA secreting cell. The area coefficient of IgA secreting cell = the area of IgA secreting cells (μm^2)/the area of the selected tissue (μm^2). All the data were expressed as mean±S.D. and analysed by one-way ANOVA using SPSS 13.0. The treatment effects were considered significant if the *p*-value was at or below 0.05.

Results

The dynamic changes of serum antibody titer

Dynamic changes of serum HI antibody titers were listed in Table 3. In general, the antibody titers of all SJZD treated groups at each time point were higher than that of Group E. The antibody titers in group B and group C were significantly higher than those of group E ($p < 0.01$) on days 40 and 60. Meanwhile, the average antibody titers were increased about two titers. On days 30 and 50 the antibody titers of group B and group C were significantly higher than those of group E ($p < 0.05$). There was no significant difference in the antibody titers at most time points between group A and group E.

Table 3: ND antibody titer in different experimental groups

Group	20d	30d	40d	50d	60d
A	5.3±0.71 ^b	7.1±0.89 ^B	7.3±1.14 ^b	7.4±0.54 ^B	6.2±0.45 ^C
B	5.8±0.83 ^b	7.8±0.41 ^{Bb}	8.2±0.83 ^{Ca}	8.1±1.14 ^{Ba}	7.2±0.83 ^{Bb}
C	5.6±1.14 ^b	7.9±0.75 ^{Bb}	8.6±0.89 ^{Ca}	7.8±0.83 ^{Ba}	8.1±0.71 ^{Bb}
D	4.0±0.71 ^a	3.7±0.44 ^A	3.3±0.55 ^A	2.8±0.50 ^A	2.0±0.64 ^A
E	5.2±1.30	6.7±0.81 ^{Ba}	6.4±0.54 ^B	6.5±1.14 ^{Bb}	5.8±1.10 ^{Ca}

Note: Values in the same column with different letters are significantly different, lowercase means $P < 0.05$, majuscule means $P < 0.01$.

Table 4: The influence of SiJunZi Decoction on the IgA secreting cells in the intestine of chicken

Tissues	Day	A	B	C	D	E
Duodenum	20	1.59±0.15 ^{Aac}	1.49±0.21 ^{ac}	1.52±0.13 ^{Ac}	1.13±0.093 ^{Bbc}	1.23±0.11 ^b
	40	4.42±0.17 ^A	4.34±0.84 ^A	4.44±0.28 ^A	2.57±0.13 ^B	3.01±0.13 ^B
	60	6.56±0.50 ^A	7.78±1.45 ^A	7.51±1.23 ^A	4.01±0.56 ^B	4.79±0.56 ^B
Jejunum	20	1.01±0.23	1.19±0.16 ^a	1.19±0.21 ^a	0.83±0.16 ^b	1.02±0.95
	40	2.22±0.33 ^a	2.29±0.14 ^a	2.31±0.11 ^a	1.40±0.046 ^b	1.82±0.18 ^c
	60	4.15±0.33 ^A	4.19±0.15 ^A	4.16±0.12 ^A	3.10±0.16 ^B	3.99±0.12 ^A
Peyer's Patch	20	0.82±0.21	0.77±0.19	0.96±0.16 ^a	0.52±0.16 ^b	0.73±0.23
	40	1.78±0.17 ^{ac}	1.78±0.23 ^{ac}	1.87±0.18 ^a	1.23±0.11 ^b	1.52±0.27 ^{bc}
	60	2.69±0.64	3.18±1.08 ^{ab}	3.49±0.17 ^{Aa}	1.92±0.14 ^{Bc}	2.26±0.42 ^{bc}

Note: Values in the same column with different letters are significantly different, lowercase means $P < 0.05$, majuscule means $P < 0.01$.

Table 5: The weights of chickens in different experimental groups during the administration of SJZD

Group	Grams on day 12	Grams on day 19 PT	p-Value
A	111.0±3.61	177.1±5.57	NS
B	109.6±4.73	182.8±12.53	ns
C	110.6±5.81	177.6±11.68	ns
D	110.3±3.51	176.3±16.52	ns
E	111.1±3.91	169.7±10.59	ns

PT: Post-SJZD treatment. NS: Not significant (compared to the relevant treatment or control groups)

The area coefficient changes of IgA secreting cells in duodenum, jejunum and Peyer's Patch.

In each fragment of intestine samples, IgA secreting cells were recognized as lymphocytes by their characteristic morphology: rounded, with a nucleus surrounded by a ring of yellow-brown cytoplasm. These cells were present in the lamina propria of villi in the duodenum and jejunum surrounding the glands (Figure 1 A-D). In the duodenum, the area coefficient of IgA secreting cells of group A and group C was increased significantly than that of group E ($p < 0.05$) on day 20. The area coefficients of all SJZD treated groups were increased significantly than that of group E ($p < 0.01$) on days 40 and 60. On day 40, the area coefficient of IgA secreting cells in the jejunum of all SJZD treated groups was increased significantly than that of group E ($p < 0.05$). The area coefficient of IgA secreting cells in the Peyer's Patch, only higher in group A than that of group E ($p < 0.01$) on days 40 and 60 (Table 4).

The IL-2 and IFN- γ mRNA level changes in jejunum

Transcription of IL-2 mRNA and IFN- γ mRNA in the jejunum was evaluated on days 20, 40 and 60 by RT-PCR. Compared with group E, high levels of IL-2 mRNA responses were detected in all vaccination groups with Sijunzi Decoction treatment. The IL-2 mRNA levels of all SJZD treated groups were increased significantly than that of Group E ($p < 0.01$ and $p < 0.05$) on days 20 and 60 respectively. The IL-2 mRNA levels of group A and group B were increased significantly than that of group E ($p < 0.05$) on day 40. In addition, SJZD increased IFN- γ expression in the group B ($P < 0.05$) and significantly increased IFN- γ mRNA in the group C ($P < 0.01$) on day 20. At this time point SJZD increased IFN- γ expression in a dose-dependent manner in all groups. The mRNA level of IFN- γ in group B was significantly higher than that of group E ($p < 0.05$) on day 40, but on 60 days old, The expression of IFN- γ mRNA was decreased and became similar to the group D and group E (Figures 2 and 3).

Safety tests

All chickens used for testing the safety of SJZD remained clinically healthy after the administration of SJZD. There was no difference observed in the mean weights of chickens treated with SJZD, the mean weight of the high dose SJZD group being 110.6 at the beginning and 177.6 g at the end of the administration, respectively. By comparison, the mean body weight recorded for the group D was 110.3 and 176.3 g, respectively (Table 5). Four chickens did not survive during the entire period of the experiments, equally distributed into every group except group B, giving an overall survival rate of 97.3% (Figure 4). It is indicated that there was no significant SJZD treatment effect on chicken survival rate. Therefore, these results suggest it is safe to use SJZD in chickens in this study.

Discussion

The immune response to NDV has been studied extensively. A number of NDV-adjuvant preparations have been investigated in animals (Zhang et al., 2007; Guo et al., 2009) and the specificity of the immune response is an integral parameter for assessment of novel adjuvant preparations (Marciani, 2003). Merz et al. (1981) reported that both humoral and cellular immune responses played important roles in the host's defense against NDV infection. The herbal formula Sijunzi decoction has been used in ancient China as a tonic and as an immunoenhancer in modern times (Zhang ed., 1990). Therefore, we studied the immunopotentiating effects of the formula. For humoral immune responses, serum antibody titer and IgA secreting cells were investigated. For cellular immune responses, IFN- γ mRNA and IL-2 mRNA were studied.

Previous studies have shown that the HI antibody is directly effective against NDV in chickens (Maas et al., 2003; Roy et al., 1999) and therefore antibody titer responses have been used as indicators of humoral immune status of birds (Kong et al.,

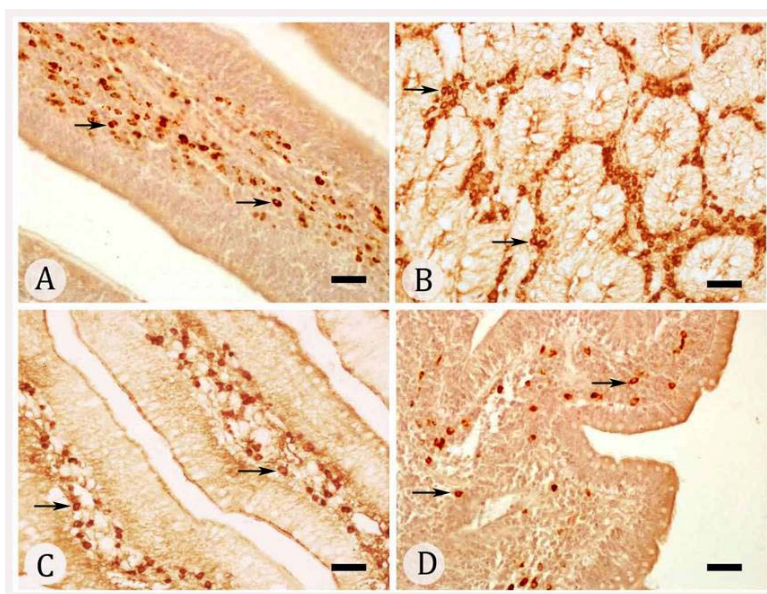


Figure 1: A. IgA secreting cells in duodenum. B. IgA secreting cells around the intestinal glands. C. IgA secreting cells in jejunum. D. IgA secreting cells in Peyer's Patch. The IgA secreting cells were pointed by the arrows. Scale bar=30µm.

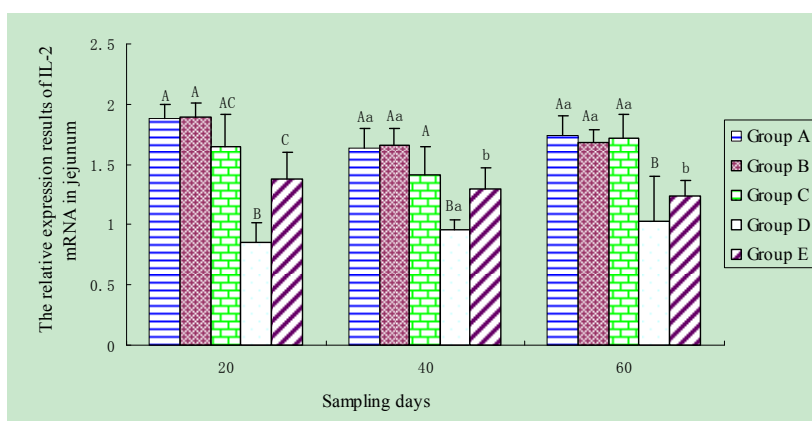
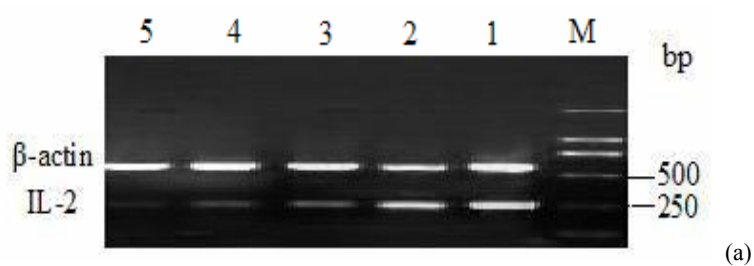
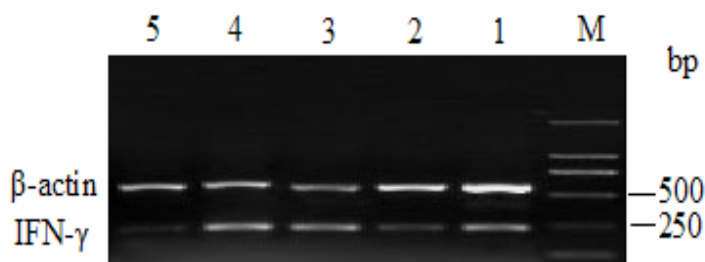


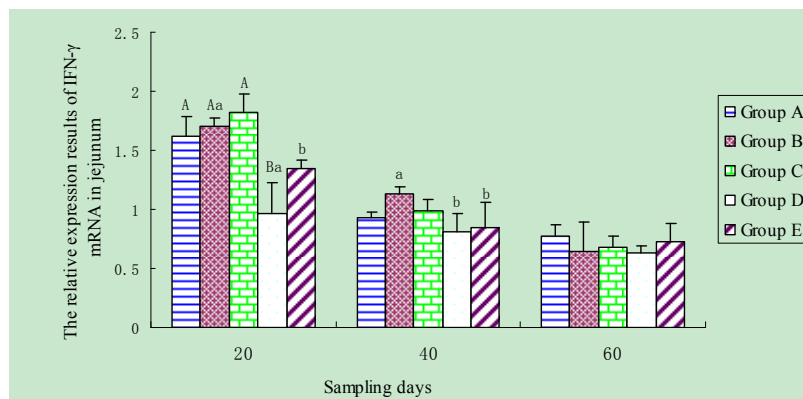
Figure 2: The RT-PCR electrophoresis photo (a) and analysis results (b) of SiJunZi Decoction on the level of IL-2 mRNA

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expression. M: 2000bp ladder of DNA markers; Lane 1: The samples of High(0.5 g/ml); Lane 2:Medium(0.25 g/ml); Lane 3: Low(0.125 g/ml) dosages of Sijunzi Decoction; Lane 4: ND; Lane 5: the control.



(a)



(b)

Figure 3: The RT-PCR electrophoresis photo (a) and analysis results (b) of SiJunZi Decoction on the level of IFN- γ mRNA expression. M: 2000bp ladder of DNA markers; Lane 1: The samples of High(0.5 g/ml); Lane 2:Medium(0.25 g/ml); Lane 3: Low(0.125 g/ml) dosages of Sijunzi Decoction; Lane 4: ND; Lane 5: the control.

2004). In this experiment, the anti-ND virus HI antibody titers in most treatment groups were higher than that of the control group at nearly all time points. From day 30 to day 40, the titers in each group reached higher levels. In all SJZD treated groups, the titers reached 7.8 to 8.6 log₂, whereas in the control group, the titers reached 6.4 to 6.7 log₂. On day 60, the titer in the control group dropped to 5.8 log₂. Whereas in SJZD treated groups, the titers dropped to 6.2 and 8.1 log₂. These findings indicate that at medium and high dosages SJZD could promote specific antibody production earlier and maintain it longer, and thus improve the immune effect of the vaccine.

The IgA secreting cells in the laminal propria tissue of intestine are the important effect molecules in protecting mucosal surfaces. Now the changes of IgA secreting cells in intestine are one of the standards to estimate intestine mucosal immunity (Zhang et al., 2007). The results of the experiment showed that the areas of IgA secreting cells of all SJZD treated groups were higher than that of the ND vaccine-only Group in the whole immune period. Consequently, it can be concluded that SJZD could stimulate the formation of IgA secreting cells in the local mucosal and enhance the local immune response of the small intestine. The immunomodulatory properties of adjuvant are well-documented. Many substances have been shown to act as vaccine adjuvants (Singh and O'Hagan, 2003; Nugent et al., 1998). Furthermore, the majority of which have only been used in admixture with antigen. Most significantly in this study, the antigen (ND) and adjuvant (SJZD) were not administered in admixture but at separate times according to a 'Two-Time Immunization Method (TTIM)' developed in our laboratory. The ability of oral SJZD to enhance mucosal immunized antigen suggests an alternative mode of action as conventional adjuvants are thought to rely on physicochemical interactions including the maintenance of antigenic conformation and depot related effects that stimulate immune activation (Yang et al., 2005; Schijns and Tangerås, 2005).

In addition to antibodies, cell-mediated immunity also plays an important role in immunity against virulent ND virus

(Zhang et al., 2008). In recent years, numerous studies had demonstrated that a lot of Chinese herbal medicine could improve the expression of cytokines so as to enhance the immunity (Yang et al., 2008; Chu et al., 2005; Wang et al., 2006a). However, these studies were focused on the changes of cytokine mRNA transcription of peripheral lymphocytes and monocytes after stimulation in the short term. In our experiments, total RNA was extracted from jejunum directly, then changes of IL-2 mRNA and IFN- γ mRNA was detected. The mRNA expression has become more sensitive and more directly in reflecting local immune responses following oral SJZD treatment of NDV-immunized chickens. High levels of IL-2 mRNA responses were detected in all treated groups. Additionally, The change of IFN- γ mRNA was detected in initial stage. Furthermore, the results increased linearly when the level of SJZD increased. But in late immune stage, the change of IFN- γ mRNA was not detected. We assumed that the expression of IFN- γ mRNA was higher in initial immune stage, and then the mRNA was rapidly translated into protein. So the IFN- γ mRNA transcription was decreased in the late stage of immunization.

At present, there were only a small number of reports about the application of Chinese herbal medicine as a mucosal adjuvant to improve the immunity. This study reports, for the first time to our knowledge, the immunostimulatory capacity of the complete SJZD preparation as a mucosal adjuvant. SJZD contains extracts from multiple herb extracts that exhibit immunostimulatory and may partly explain this observed SJZD effect. Many studies have demonstrated application of SJZD on the base of enteric nutritional therapy can lessen the degree of post- operational stress and inflammatory response, and enhance the immune function of patients (Cai et al., 2008; Wu and Xuan, 2007; Liang et al., 2005). Rivera et al.(2003a; 2003b) reported that the synergistic effect of aluminium hydroxide and ginseng was observed. *Poria cocos* was reported to stimulate a number of immune response parameters including NO production in macrophage *in vitro* and *in vivo* as well as IFN- γ and TNF- α (Lee and Jeon, 2003; Chen and Chang, 2004). *Glycyrrhiza uralensis* saponins (GLS) isolated from *Glycyrrhiza uralensis* were reported to show a slight haemolytic effect and to enhance significantly the specific antibody and cellular response against OVA in mice (Sun and Pan, 2006). In this study, the induction of immune responses in both systemic and mucosal immunity indicates that the combination of bioactive vaccine within SJZD acts synergistically to produce an overall stimulatory effect on immune cell activation.

In conclusion, our results showed that SJZD had effects on enhancing humoral and cellular immune responses to NDV-immunized chickens. Taken together with its natural origin, without lethal toxicity to humans and animals, and longstanding use as folk medicines, SJZD may be useful as a novel mucosal adjuvant that improves the effectiveness of chicken vaccines or provides optimal protection against common infections in chickens.

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