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Immunoregulation of *Lycium barbarum* Polysaccharide in Vaccinated Chickens

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Abstract: The objective of the present study was to evaluate the effects of *Lycium barbarum* Polysaccharide (LBP) on immune responses in vaccinated chickens. A total of 600 Hy-Line Brown chickens aged 15 days old were randomly divided into four groups with three replicates per group and fifty chickens per replicate and all the chickens were injected with Newcastle Disease (ND) vaccine. Three experimental groups of chickens were injected with LBP 20, 10 and 5 mg kg^{-1} (LBP_H, LBP_M and LBP_L) and the control group were injected with equal dose normal saline (0.09% NaCl), respectively per day for 7 days. On the 7, 14, 21, 28, 35 and 42 days after vaccination, ten chickens were sampled randomly from each group and the serum was seperated for the determination of NDV-HI antibody titers by Micro Method. On day 10, 20, 30, 40 and 50 after vaccination, blood samples from 5 chickens per group were collected to separate lymphocyte and determine the peripheral blood T lymphocyte proliferation with Methyl Thiazolyl Tetrazolium (MTT) Method. The content of CD4+ and CD8+ T cells were tested by using flow cytometry with Double Color Staining Method. The IL-2 levels were determined by ELISA Method and the whole body, bursa of fabricius and thymus were weighted for calculation of immune organ index. The results showed that LBP (10 and 20 mg kg^{-1}) could significantly raised the ratio of CD4+ and CD8+T lymphocyte ($p<0.01$) and the production of IL-2. It also significantly enhanced the ND antibody titers, promoted the proliferation of peripheral blood T lymphocyte and increased immune organ indexes ($p<0.01$). These results indicated that LBP had significant immunoregulation functions of ND vaccine in chickens.

Key words: *Lycium barbarum* polysaccharide, vaccinated chickens, antibody, IL-2, T lymphocyte, ratio of CD4+ and CD8+T lymphocyte, immune organs, China

INTRODUCTION

Lycium barbarum was the ripe fruit of Solanaceae. It was a kind of traditional Chinese Medicine and named as rubies which was first recorded in Sheng Nong's Herbal Classic. Modern pharmacological research showed *Lycium barbarum* Polysaccharide (LBP) was the main active ingredients of *Lycium barbarum* which could adjust immunity, delay ageing, resist tumour, reduce blood fat, decrease blood sugar, fight fatigue, protect the reproductive system and resist lack of oxygen. Various functions of LBP came from its immunoregulation to bodies. A number of experiments showed that LBP could exert regulate functions on immune system in various ways and many aspects.

It could not only activate T, B lymphocytes to cause body's specific immune response but also activate non-specific immune response by activating Macrophages (MO), Natural Killer cells (NK), Dendritic Cells (DC), etc. and inducing cell factors secretion. Based on this function it could play the role in resisting tumour delaying ageing and radioresistance, etc. (Yongjie *et al*., 2010; Guiju and Pingguo, 2010; Cheng and Kong, 2011).

The immunoregulation of LBP in vaccinated chickens has not been reported at present. This experiment extracted polysaccharide composition from *Lycium barbarum* and combined vaccine in chickens.

By means of measuring the dynamic changes of serum Newcastle Disease (ND), Hemagglutination Inhibition (HI) antibody titers, the peripheral blood T lymphocyte proliferation the levels of cell factors IL-2, the content of CD4+ and CD8+T cells and the immune organ index, evaluate the immune effect of LBP to the vaccine and investigate the mechanism of enhancing the effect of vaccine immune and provide the theory basis for the development of polysaccharides.

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MATERIALS AND METHODS

Medicines preparation: LBP was extractd by water extraction and alcohol precipitation method from *Lycium barbarum* (location: Ningxia province). The content of polysaccharide was detected to be 80.25% with anthranone sulfuric acid method. LBP were dissolve in RPMI-1640 (HyClone laboratories, Logan, UT) without calf serum to make into different concentrations. Sterilize with microporous membrane filter (0.22 μm, Ireland Millex company) and store it at 4°C for using.

Experimental animals grouping and processing: A total of 600 Hyline brown chickens without being vaccinated (purchased from Chicken Farm of Hebei Zhangjiakou of China and fed with full price ration) were isolated to be fed to 15 days old (average body quality was 100.23 g) and injected with the same Newcastle disease vaccine Lasota plant 1.0 mL. At the same time these chickens were randomly divided into four groups with three replicates per group and fifty chickens per replicate. Experimental chickens were injected with LBP 20, 10 and 5 mg kg^{-1} (LBPH, LBPM and LBPL group) and the control group were injected with equal dose normal saline (0.09% NaCl), respectively per day for 7 days.

ND HI antibody titer measurement: Blood samples (5 mL) were collected randomly from wing venous of 10 chickens from each group on day 7, 14, 21, 28, 35 and 42 after vaccination. Centrifuge the serum for 5 min at 1000 r min^{-1} to determine the NDV-HI antibody titer by Micro Method (Thekisoe *et al*., 2004).

Measurement of proliferation of peripheral T lymphocyte: On day 10, 20, 30, 40 and 50 after vaccination, blood samples (5 mL, citric acid sodium anticoagulation) were collected from 5 chickens per group to separate lymphocyte and determine the proliferation of peripheral blood T lymphocyte with MTT Method (Li *et al*., 1996; Wang *et al*., 2005; Kong *et al*., 2004).

The anticoagulation blood samples levitation liquid was slowly added to a 10 mL centrifuge tube along the wall to lie on top of 1 mL percoll lymphocyte separation medium (pharmacia company) that was already in the tube and the test tube was stoppled with rubber. After being centrifuged at 1500 rpm for 15 min, the cloudy low density cell layer (second layer) was pipetted into another centrifuge tube and washed with RPMI-1640 medium twice. The final cell pellet was resuspended in RPMI-1640 medium and cell viability (>95%) was checked by thiazolyl blue viability assay. The final cell suspension was adjusted to 2.0×10^6 cells mL^{-1}.

Total 50 μL lymphocytes and 50 uL PHA were successively added and proliferated in 96 well (Costar Company of America) tissue culture plates. The experiment was conducted with 3 replicates. The plates were incubated in a 5% CO_2 incubator (MCO-18AIC, Sanyo Corporate, Japan) at 39°C for 48 h. At the end of incubation, 10 μL of 5 mg mL^{-1} MTT (ultra pure grade, Amresco®, repackaged by Beijing Kehaoze Biologic Technology Co., Ltd.) was added into each well.

After another 4 h of incubation at 37°C, DMSO (ACS grade, Amresco®, repackaged by Beijing Kehaoze Biologic Technology Co., Ltd.) was added to stop the reaction. The plates were read at 570 nm with a microplate reader (Model 680, Bio-Rad Laboratories, CA, USA) and OD values were recorded. Higher = OD570 values represented higher levels of lymphocyte proliferation (Mosmann, 1983).

T lymphocyte subsets measurement: On day 10, 20, 30 and 40 after vaccination, blood samples (5 mL, citric acid sodium anticoagulation) from 5 chickens per group were collected to separate lymphocyte and the final cell suspension was adjusted to 2.0×10^6 cells mL^{-1} with PBS. Lymphocytes suspension and marked Mab (RC-CD4+-mAbRC-CD8+-mAb, RC-IgG-HRP RM-IgG-FITC) bought from Shenzhen Jingmei Biological Engineering Limited company, China were respectively added to the tube and the content of CD4+ and CD8+T cells were tested by using flow cytometry FACSAria Becton, Dickinson and Company with Double Color Staining Method.

IL-2 of peripheral blood lymphocyte measurement: Blood samples were collected to separate lymphocyte from 5 chickens per group on day 10, 20, 30, 40 and 50 after vaccination and 100 uL lymphocytes were successively proliferated in 96 well tissue culture plates. Each group had four replicates. The plates were incubated in a 5% CO_2 incubator at 39°C for 72 h. At the end of incubation, the IL-2 levels were determined by IL-2 kit (Sigma; operation steps according to the instructions).

Measurement of immune organ index: On day 10, 20, 30, 40 and 50 after vaccination, 5 chickens per group were sacrificed and the hole body, bursa and thymus were weighted for calculation of immune organ index (Li, 1999):

$$\text{Immune organ index} = \frac{\text{Organ wet weight}}{\text{Hollow live weight}}100\%$$

Statistical analysis: A Duncan's multiple analysis was done with SPSS 11.5 Statistical Software. Results were expressed as mean±SD. Experiment were considered significant difference at $p<0.05$.

RESULTS

Effect of LBP on ND HI antibody titers: The antibody titers of the experimental groups were higher than those of the control group in all detection point of antibody. The antibody titers to ND of LBP_M and LBP_H groups were remarkably different from those of the control group ($p<0.05$) after being vaccinated for 7~14 days.

The antibody titers of LBP_L were higher than those of the control group too but not significantly ($p>0.05$). After being vaccinated 21~35 days, the antibody titers of LBP_M and LBP_H groups were significantly higher than those of the control group ($p<0.01$) and reached the peak of antibody titers on 35th day and declined slightly on day 42 but still had significant difference with the control group ($p<0.05$) (Fig. 1).

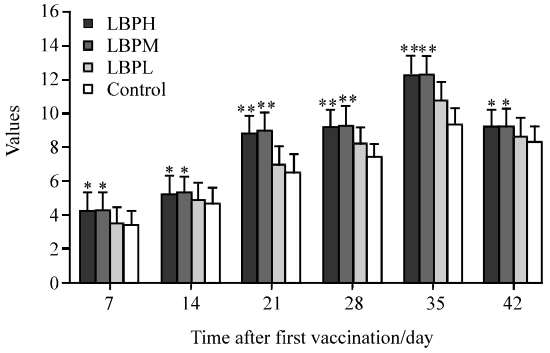

Fig. 1: Effects of LBP on ND antibody titer in chicken (log2). Bars marked with ** are highly significant compared to the control (0 μg mL^{-1}) ($p<0.01$), marked with * are significant compared to the control ($p<0.05$)

Effect of LBP on the proliferation of peripheral blood T lymphocyte: The OD_{570} values of experimental groups were higher than that of the control group after being vaccinated 10 days but not significantly ($p>0.05$) after being vaccinated 20~40 days, the OD_{570} of LBP_M group was significantly different from that of the control group ($p<0.01$) and reached the peak of OD_{570} on day 42 after being vaccinated; LBP_H group had significant difference with the control group ($p<0.01$) after vaccinated 40 days; LBP_L group had distinct difference with the control group ($p<0.05$) after vaccinated 30~40 days. The OD_{570} of experimental groups declined slightly after being vaccinated 50 days but LBP_M and LBP_H groups still had significant difference with the control group ($p<0.05$) or ($p<0.01$) (Fig. 2).

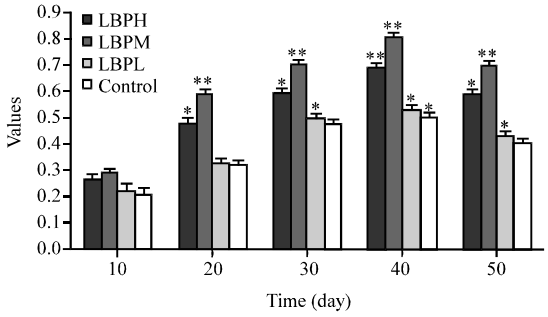

Fig. 2: Effects of LBP on proliferation of peripheral blood T lymphocyte in chicken (OD_{570})

Effect of LBP on T lymphocyte subsets: The ratios of CD4+/CD8+ T lymphocyte of experimental groups were higher than that of the control group after being vaccinated 10 days but only LBP_M group had significant difference with the control group $p<0.05$ LBP_M group were increased much more than the control group after vaccinated 20~40 days and especially they were significantly different from the control group after vaccinated 30 days ($p<0.01$) (Fig. 3).

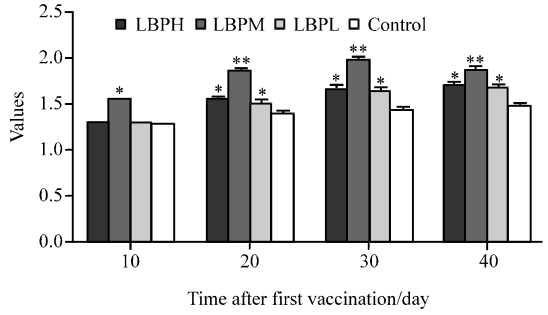

Fig. 3: The dynamic changes of the ratio of CD4+/CD8+T lymphocytes

Effect of IL-2 of peripheral blood lymphocyte: The IL-2 of peripheral T lymphocyte in chickens were increased obviously after being vaccinated 10 days in all experimental groups; LBP_M group were significantly different from the control group after vaccinated 10~50 days ($p<0.01$), LBP_H and LBP_L groups had distinct difference with the control group after vaccinated 20~50 days $p<0.05$ (Fig. 4).

Effect of immune organ index of chicken: LBP groups could significantly enhance the functions of central immune organs such as thymus and bursa of fabricius, especially to thymus. Thymus organ index of LBP_M group had significant difference compared with the control group after being vaccinated 10~20 days $p<0.05$ and was promoted much more than the control group after being vaccinated 30~50 days ($p<0.01$) and reached the peak of

Table 1: Effects of LBP on immune organ index in chicken

Organs	Group	Time after the first vaccination/days				
		10 days	20 days	30 days	40 days	50 days
Thymus	LBP_H	3.770±0.005	3.989±0.013	4.585±0.020*	4.785±0.020*	4.989±0.018*
	LBP_M	4.285±0.013*	4.289±0.020*	5.795±0.012**	6.595±0.012**	5.997±0.013**
	LBP_L	3.618±0.015	3.935±0.014	4.354±0.016*	4.363±0.016*	4.975±0.014*
	Control	3.532±0.151	3.762±0.230	4.081±0.127	3.929±0.265	4.273±0.181
Bursa	LBP_H	1.870±0.013	2.083±0.015*	2.985±0.017*	2.385±0.011	2.489±0.016
	LBP_M	2.285±0.021*	2.289±0.010*	3.295±0.018**	2.695±0.015*	2.597±0.011*
	LBP_L	1.718±0.014*	1.935±0.013*	2.654±0.105	2.354±0.012	2.375±0.201
	Control	1.690±0.251	1.871±0.012	2.542±0.122	2.283±0.012	2.083±0.282

The value marked with ** are highly significant compared to the control (0 μg mL^{-1}) ($p<0.01$), marked with * are significant compared to the control ($p<0.05$)

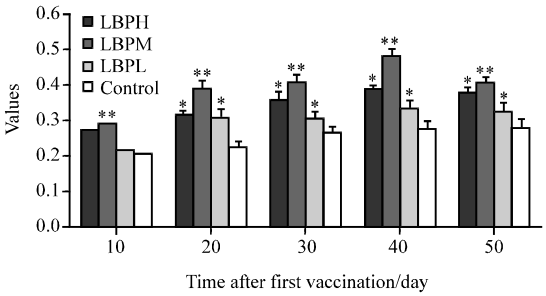

Fig. 4: Effect of LBP on the IL-2 activity of peripheral blood T lymphocyte in chicken

thymus organ index on day 40 after being vaccinated; LBP_H and LBP_L groups were significantly different from the control group after vaccinated 30~50 days, too $p<0.05$. The influence of bursa of fabricius organ index of all LBP groups had been improved in some way but LBP_M group were significantly different from the control group after vaccinated 30 days only($p<0.01$) and reached the peak of bursa of fabricius organ index (Table 1).

DISCUSSION

Effects of LBP on cellular immunity zin chickens: T lymphocytes were the important cells of immune regulation and effector cells of the body. They had the largest number and the most complex functions. T lymphocytes could act on the target cells directly and play a very important role in cellular immunity and the function of immune system would disorder extensively if the total number was not enough or subsets were unbalanced.

The recent research showed that LBP could also regulate the proliferation of T lymphocytes and subsets of stability. Liu *et al.* (2000) reported that LBP could increase total T cells and the percentage of Th subsets. It could also improve LT rate and help to restore the lower immunity function induced by Cyclophosphamide (CTX) It could make the decreased numbers of total T cells, helper T (Th), suppress T (Ts), Th/Ts and the rate of Lymphocyte Transformation (LT) return to normal. The experimental results demonstrated that *Lycium barbarum* poysaccharide could positively regulate immunity functions. Wang and Li (2002) found that LBP could obviously promote T lymphocyte proliferation by drenching a mouse with 25 mg/kg/days. He *et al.* (2005) reported that LBP could increase the numbers of CD4+, CD8+ T cells and the proliferation of CD4+ T cells and elevate the ratio of CD4+/CD8+ in peripheral blood. Wang *et al.* (2000) reported that *Lycium barbarum* and Rhizoma atractylodis could remarkably enhance the effects on the expression of IL-2 receptor and have immunoregulatory effects on immunologic functions. Wang *et al.* (2009) studied the effects of LBP-D on immune functions in Alloxan-Induced Diabetes mice. The results showed that LBP-D had remarkably enhancing effects on the immune functions including the enhancement of lymphocyte proliferation, the regulation in the number of T lymphocyte subsets and the improvement of the IL-1 and IL-2 levels. It could make the immuno-compromised functions of the alloxan-induced diabetes mice return to normal or near to normal.

The experimental results showed that LBP could significantly enhance the proliferation of T lymphocytes and the ratio of CD4+/CD8+ and IL-2 levels of peripheral blood lymphocyte in chickens. The proliferation and differentiation of T lymphocyte were an important stage of immune response to bodies so the degree of lymphocyte proliferation affected the immune level of bodies directly. The experimental results showed that LBP (10 and 20 mg kg^{-1}) could significantly raise the proliferation and transformation rate of peripheral blood T lymphocyte compared with the control group.

CD4/CD8 ratio of T cell subsets was an important index of T lymphocytes activity and the immunity would be enhanced if the ratio increased in the normal range, otherwise it would be weakened. This research showed that CD4+/CD8+ ratios of experimental groups were higher than that of the control group, especially LBP_M (10 mg kg^{-1}) group were higher significantly.

IL-2 was a kind of wide biological activity cytokines produced by the activated CD4+ and CD8+ T cells. It was growth factors of all T cell subsets and could promote the proliferation of activated B cells. So, IL-2 was important factors of the immune response regulation and could

participate in antibody response, hematopoiesis and tumor surveillance. This research showed that LBP could induce the production of IL-2 from peripheral blood lymphocyte in chickens significantly. LBP had widely immunity activity and could strengthen the cellular immune functions of bodies.

Effects of LBP on humoral immunity in chickens: Traditional Chinese medicine polysaccharides could significantly enhance the humoral immune level in chickens (Gu *et al*., 2005) and NDV antibody level was an important index of specific humoral immune function of vaccinated chickens with ND. The research results showed that the antibody titers of all LBP groups were higher than those in the control group; 7~42 days after the first vaccination, the antibody titers of LBP groups were higher than those of the control group, especially LBP_M group remained 1 Log2 higher than the control group during 21~35 days and the antibody titer of LBP_M group was still in 12.297 Log2 while the control group's dropped to 9.492 Log2 on day 35. It showed that proper dose of LBP could significantly increase the specificity immune response in chickens and extend the time of keeping high antibody levels and improve the effects of vaccine immune.

Effects of LBP on immune organ index of vaccinated chickens: Immune organ index was an important index of the growth and development of immune organ and could react to the immune functions of bodies as auxiliary immune detection means. Bursa of fabricius was the place where B lymphocytes could differentiate, grow and mature and the thymus was T lymphocytes. Test results showed that LBP could enhance the central immune organs thymus and bursa of fabricius index at different time, especially to the thymus. Thymus organ index of LBP_M group was promoted significantly compared with that of the control group after being vaccinated 30~50 days ($p<0.01$) and reached the peak of thymus organ index on day 40 after being vaccinated; the bursa of fabricius organ index of all LBP groups had elevated to certain degree but LBP_M group was significantly promoted much more than the control group after vaccinated 30 days ($p<0.01$) and reached the peak of bursa of fabricius organ index.

CONCLUSION

The results showed that proper dose of LBP could significantly enhance the antibody titers to ND, raise the ratios of CD4+ and CD8+T lymphocyte and the immune organ indexes and the production of IL-2 and promote the proliferation of peripheral blood T lymphocyte. These results indicated that LBP had significant immune enhancement functions on immunity of ND vaccine in chickens.

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