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Study on Immunological Activity of Iron-Zymosan

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Abstract: To investigate the immunological activity of Iron-Zymosan (IZS) extracted from iron-yeast cells. The mice were taken as research object, the expression IL-2 mRNA and IL-2R mRNA were evaluated in mice spleen cells, the amount of IgA^+ cells were assayed in intestinal mucosa and the content of IgA was detected in serum of mice. The results showed that IZS had immunoregulatory activity and could be explored as a novel iron-zymosan source in dietary supplements.

Key words: Iron-zymosan, IL-2 mRNA, IL-2R mRNA, IgA, immunological activity

INTRODUCTION

Polysaccharide is a biopolymer with wide distribution and abundant content in nature. Bacteria polysaccharides from microbial sources has drawn great attention because it has no toxicity, unique physical and chemical properties, broad applications and it not only can be separated from thallus easily but also can be realized industrialized production by deep fermentation (Tzianabos, 2000; Sletmoen *et al*., 2003). Compare with plant polysaccharide, animal polysaccharide and the others, the production cycle of microbial polysaccharide is short and it is free from season, region and plant diseases and insect pests (Wang *et al*., 2007). So, it has strong market competitiveness, broad development prospects and extensive application advantages. Yeast cell wall is surrounded with 85-90% carbohydrates and 10-15% protein and half of carbohydrates are glucan and mannan (Caric *et al*., 2002). Yeast glucan has significant roles in antiradiation, antioxidant, antiaberration, antigenotoxicity, antitumor, antiinfection and promote the wound healing and it is a kind of good biological effect regulator (Holanda *et al*., 2005; Matsuo *et al*., 2000; Cipak *et al*., 2001; Freimund *et al*., 2003; Slamenova *et al*., 2003; Khalikova *et al*., 2005; Kogan *et al*., 2005, 2008; Kogan and Kocher, 2007). Ferrum is the necessary metal trace elements for human and broadly participate in the metabolic processes, such as oxygen transportation, DNA synthesis, the electronic transfer, etc. Iron Deficiency Disease (IDD) is a worldwide nutritional disease and it is estimated that about 700 million people around the world suffer from iron deficiency. Iron is the necessary composition of biological oxidation enzymes and DNA synthesis enzymes. If the activity of iron enzyme and Fe-dependent enzyme reduced, the poor performance of the non-blood system can be caused and it will have large negative effect on the human body intelligence, the physique, the immune system, the digestive system, the labor ability and so on (Ballot *et al*., 1989). So, the iron content of body immediately influence on the growth status and metabolic process and IDD has been listed as one of four nutritional diseases by the world health organization (Lozoff, 1988). In recent years, many researches show that organic form trace elements can improve absorption efficiency of trace elements in animal bodies, reduce the other nutrients losses in feed and provide low cost and rich variety of nutrition trace elements (Hentze *et al*., 2004; Fleming, 2005).

Iron-zymosan can play double function of trace element iron and zymosan and the activity of iron yeast is higher than that of iron and zymosan, respectively. At present, the study of the iron-zymosan was not reported.

MATERIALS AND METHODS

Iron-zymosan: It is repared by Lanzhou Institute of Husbandry and Pharmaceutical Sciences of China Academy of Agricultural Sciences, China.

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Mice: The clean level, Kunming-stain, 20±2 g, from Experimental Animal Research Center, Lanzhou University, China.

Reagents: Goat-anti-mouse IgA (Sigma); SABC immunohistochemical Staining kit (Wuhan Boster Bio-Engineering Limited Company, China); DAB enzyme substrate color-developing reagent kit (MBI, American); RNAiso Reagent (TaKaRa Biotech Company); High Fidelity RNA RT-PCR Kit (TaKaRa Biotech company); the primers of IL-2R and IL-2 were synthesized by Sangon Biotech (Shanghai) Limited Company, China. Primer sequence:

IL-2R:5-CGGTTTCCGAAGACTAAA-3, 5-GTCCTTCCA-CGAAATG AT-3;
IL-2: 5-CTGGAGCAGCTGTTGATG-3, 5-CGAATTGGCACTCAAATG-3.
β-Actin: 5-GTTACCAACTGGGACGACA-3, 5-AGGCATACA-GGGAC AGCA-3

Instruments: High speed refrigerated centrifuge TGL-168 (Shanghai, China); IX50 Olympus microscope and Moditec image acquisition analysis system; DNA amplificater (MinicyclerMJ-150 Research com), AlphaInnotech Corporation, UVP-800 multiImager and Labworks 4.0 Image Analysis Software, SpectreMax M2 microplate reader (Molecular devices company of the United States), Atomicabsorption spectroscopy (VARIAN) and other common instruments.

Preparation of iron-zymosan: The yeast wall was broken through the combining method of high pressure and freeze-thaw and then the iron-zymosan was extracted by using alcohol deposit process (Liu *et al*., 1994). Iron-zymosan was removed of protein by Sevag method and purified by chromatography with Sephadex-G200 gel column (Pharmacia, sweden). The detection results by HPLC (Agilent) showed that iron-zymosan was polymerized by glucose. The determination by Atomic Absorption Spectrometer (VARIAN) showed that the content of iron ions in the yeast polysaccharide was 7.81 mg g^{-1}.

The mice were randomly divided into 5 groups: the Group I of iron-zymosan by 50 mg kg^{-1}, the Group II of iron-zymosan by 100 mg kg^{-1}, the Group III of iron-zymosan by 100 mg kg^{-1}, the Group IV of zymosan 100 mg kg^{-1} and the Group V of 30 mice each group. The mice was administrated intragastricaly according to the different require of every group, one time a day, continuing for 7 days, 10 mice were taken from each group at 7, 14 and 28th day randomly. Animal care was in compliance with recommendations of The Guide for Care and Use of Laboratory Animals (National Research Council).

Preparation of spleen cells splenic lymphocytes and cell suspension solution was prepared referring to the extraction method of splenic lymphocytes (Zhu and Cheng, 2003), the cells stained by trypan blue was detect the living, according to the experiment the quantity of living cells was more than 95% and the cell concentration was adjusted to 5×10^6 mL^{-1}.

The determination of IL-2 and IL-2R mRNA in spleen cells: the Reverse Transcription Polymerase Chain Reaction (RT-PCR) and image analysis were used to test the transcription level of IL-2 and IL-2 R mRNA in mice spleen cells.

The extraction of total RNA: The spleen cells of mice was put in 5 mL homogenate tube. The operation was proceded according to the kit manual. The precipitation was dissolved with RNase-free water, the ratio of OD260/OD280 which was measured by UV spectrophotometer was 1.75-1.85. The extract of RNA was stored at -80°C.

The cDNA synthesis was reaction system by 10 μL composing of total RNA 0.5 μL, 25 mmol L^{-1} $MgSO_4$ 2 μL, 2×reverse transcription buffer 5 μL, RNA enzyme inhibitors 0.25 μL, BcaPLUS RTase reverse transcription enzyme 0.5 μL, different reactants system primer 0.5 μL, dNTP Mixture 0.5 μL, RNase-free water was 0.75 μL.

The PCR reaction system was composed of cDNA template 5 μL, 10×PCR buffer was 5 μL, dNTP Mixture 1 μL, sense primer 2.5 μL, primer antisense 2.5 μL, Pyrobest DNA Polymerase Polymerase 0.25 μL, deionized water 28.75 μL. PCR cycle parameter as following: denaturation temperature 94°C, 30 sec; annealing temperature 60°C, 30 sec; extensions temperature 72°C, 2 min. Total cycle was 31 times, 72°C for 7 min. It was stored at 4°C.

Agarose gel (1.5%) electrophoresis was carried on for the RT-PCR products, the absorbancy of every stripe on the glue was quantified by scanning and the β-actin was chosen as external standard at the same time.

The change of the count of IgA^+ cells in small intestinal mucosa. On the 7th, 4th and 28th day, the mouse jejunum by 2~3 cm was taken out, the intestinal content was washed away with new configuration of saline rapidly and then put in paraformaldehyde phosphate buffer (40 g L^{-1}, pH 7.4) to be fixed. According to the routine method, the tissues were dehydrated, cleared and embedded by paraffin, then the tissues were cut into slices continuously, the thickness of slices were 5 μm. One tissues slice was taken out every 10 pieces of slices. The tissues slices were stained immunohisto-chemically according to Streptavidin Biotin Complex (SABC) Method, the number of IgA^+ cells in small intestinal mucosa was observed and compared with the control group.

The IgA^+ cells were stained by brown or dark brown under light microscopy. In negative control, 0.01M PBS was used instead of first antibody. The average light density meant the average depth of color in the tissues and the higher the Optical Density (OD) was the more the number of cells of small intestinal mucosa IgA^+ cells.

The calculation of IgA^+ cells: Two slices were randomly selected in each specimen, five vision fields was chosen under the light microscopy by 400 times, the positive cells were totalized, the images of the selected vision were collected with computer image analysis system and the average OD values of the positive signal was tested by the image analysis software of Moti Image Advaneed 3.0.

The detection of IgA content in serum: The experiment was divided into three stages by 7th, 14th and 28th days. At every experimental stage, 10 mice of each group were killed to collect blood and the serum was separated to determine the content of IgA. The IELISA Method was adopted to detect the content of immunoglobulin IgA in serum. According to the operation of the kit. As chromogenic reaction stopped, the OD of samples were detected by microplate reader at 492 nm, each sample was set three parallel hole at the same time.

Data statistics and analysis: The data was done by one-way Analysis of Variance (ANOVA) followed by LSD-t post hoc test for multiple comparisons (Computer Statistical Package, SPSS 11.0). Results were expressed as mean±SD and differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The expression of IL-2 and IL-2R mRNA: The reverse transcriptase amplification product of IL-2 mRNA and IL-2 RmRNA were scanned and quantified with the absorbance by using RT-PCR and quantitative techniques and the absorption of β-actin was used as reference in order to calculate absorbance relative value of the expression of IL-2mRNA; IL-2RmRNA (Table 1 and 2).

The expression of IL-2 mRNA in each group has no significant difference on 7th day. The expression changed and increased with the increase of dosage on 14th day. In Group II and III, the express of IL-2 mRNA had extremely significant difference ($p<0.01$) compared with control group and significant difference ($p<0.05$) compared with Group I and IV between Group I and V it had significant difference ($p<0.05$). On 28th day, the expression was lower than on 14th day but in Group II it was till significant difference ($p<0.05$) and in Group III was extremely significant difference ($p<0.01$) compared with Group V in Group III it had obvious difference ($p<0.05$) compared with Group I and IV.

Table 1: The products of IL-2 mRNA in splenic cells

	Days		
Groups	7	14	28
I	13.1±0.93	$23.9±3.17^{b}$	$20.2±2.27^{c}$
II	13.6±0.84	$30.3±2.26^{aA}$	$25.7±1.87^{b}$
III	14.0±0.73	$32.1±0.18^{aA}$	$29.1±2.16^{aA}$
IV	13.8±0.24	$20.4±1.79^{b}$	$19.4±0.43^{c}$
V	13.8±2.52	$14.2±4.72^{cB}$	$14.1±1.49^{cB}$
β-actin	70.2±1.86	-	-

Table 2: The expression of IL-2R mRNA in splenic cells

	Days		
Groups	7	14	28
I	36.2±2.02	$36.2±2.02^{b}$	$48.9±4.26^{b}$
II	36.9±2.12	$36.9±2.12^{aA}$	$59.8±3.34^{aA}$
III	40.1±5.71	$64.2±2.26^{aA}$	$61.9±1.08^{aA}$
IV	36.7±1.09	$45.1±3.17^{b}$	$43.4±1.13^{b}$
V	34.3±3.06	$34.0±2.41^{cB}$	$35.1±1.37^{bB}$
β-actin	70.9±2.13	-	-

In the same column, values with different lowercase superscripts mean significant difference ($p<0.05$), values with different capital letter superscripts mean significant difference ($p<0.01$)

Compared with Group IV and V, the expression of IL-2R mRNA in Group III increased slightly and had no significant difference among the groups on 7th day. The expression in other group except Group V rose with the increase of dosage, Compared with Group V, it had significant difference ($p<0.05$) in Group I and IV and it had extremely significant difference ($p<0.01$) in Group II and III; in Group II and III, it had significant difference ($p<0.05$) compared with Group I and IV on 14th day. The expression in each group except the Group V on 28th day began to fall but no significantly compared with that on14th day. The extent of decrease in Group I was larger than hat in Group II and III in Group II and III it still had significant difference ($p<0.05$) compared with Group I and IV and had extremely significant difference ($p<0.01$) compared with Group V.

The count of IgA^+ cells in small intestinal mucosa: Immune-histochemical results showed that the IgA positive cells distributed in proper mucous membrane, the positive granules located in the cytomembrane and cytoplasm and were dark brown. The count of IgA^+ cells and the values of average OD were shown in Table 3.

The count of IgA^+ cells began to increase on 7th day but not obvious among groups. On 14th day the count of IgA^+ cells in Group (I-IV) increased significantly ($p<0.01$) compared with Group V but there existed no obvious difference among the Group I-IV. Compared with that on the 7th days, The count of IgA^+ cells in each group

Table 3: The count of IgA^+ cells and the values of average light density

	Days					
	7th		14th		28th	
Groups	IgA^+	IgA^+OD	IgA^+	IgA^+OD	IgA^+	IgA^+OD
I	7.89±0.51	0.14±0.05	$16.29±1.24^{A**}$	$0.31±0.41^{A**}$	$15.25±0.91^{A**}$	$0.28±0.13^{A**}$
II	8.61±0.65	0.17±0.01	$19.34±1.97^{A**}$	$0.37±0.29^{A**}$	$18.34±1.27^{A**}$	$0.35±0.89^{A**}$
III	8.85±0.17	0.19±0.24	$20.01±1.21^{A**}$	$0.39±0.27^{A**}$	$18.88±1.07^{A**}$	$0.37±0.12^{A**}$
IV	7.91±0.23	0.15±0.03	$16.41±1.24^{A**}$	$0.32±0.41^{A**}$	$15.08±0.11^{A**}$	$0.27±0.75^{A**}$
V	7.64±0.34	0.12±0.08	$7.60±0.47^{B}$	$0.12±0.09^{B}$	$7.61±0.38^{B}$	$0.12±0.15^{B}$

In the same column, values with different lowercase superscripts mean significant difference ($p<0.05$), values with different capital letter superscripts mean significant difference ($p<0.01$). In comparison with the column of 7th days, the data with * shows significant difference ($p<0.05$), the data with ** shows extremely significant difference ($p<0.01$)

Table 4: The content of SIgA in serum

	Days		
Groups	7	14	28
I	$2.3612±0.0512^{a}$	$2.6741±0.0015^{a}$	$2.4030±0.0141^{a}$
II	$2.5831±0.0105^{a}$	$3.2021±0.0103^{a}$	$2.5187±0.0047^{a}$
III	$2.6013±0.0064^{a}$	$3.3946±0.0056^{a}$	$2.7021±0.0038^{a}$
IV	$2.4033±0.0018^{a}$	$2.6671±0.0213^{a}$	$2.4865±0.0039^{a}$
V	$1.8932±0.0438^{b}$	$1.9061±0.0327^{b}$	$1.9153±0.0107^{b}$

In the same column, values with different lowercase superscripts mean significant difference ($p<0.05$), values with different capital letter superscripts mean significant difference ($p<0.01$)

increased greatly except Group V ($p<0.01$); On 28th day, The count of IgA^+ cells reduced but still obviously higher ($p<0.01$) than that on 7th day, The count has no significant difference compared with that on 14th day. The change of the average was almost the same as OD of the count.

The content of SIgA in serum: The iron-zymosan and zymosan has different effecton SIgA in mice serum. The detailed results were shown in Table 4.

The content of SIgA in serum had no significant difference among the Group (I-IV) on 7th day but had significant difference compared with the Group V ($p<0.05$). The secretion of SIgA increased on 14th day, the difference of the content of SIgA in serum between each group was not obvious and compared with that on the 7th day, there existed no obvious difference. The content of SIgA decreased on 28th day, it had no significant difference among the Groups (I-IV) and but had significant difference ($p<0.05$) compared with Group V. The content of SIgA in serum has no significant difference compared with 7th day and 14th day.

The yeast glucan is one of the main active ingredients of zymosan and is a good biological regulator (Bohn and BeMiller, 1995), it could regulate and strength the immune function of the body by stimulating immune system. In 1986, Mat suzaki discovered that β-(1, 3)-D-glucan played an important role in antitumor, β-(1, 3)-D-glucan could produce the following immune response: improve phagocyte of macrophages (Williams *et al*., 1996) increase the body immune function, strengthen bactriostasis of complement system in blood plasma of higher mammals, promote differentiation of T cells and B cells, secretion of Interleukin-1 and Interleukin-2 (Sandula *et al*., 1995; Estrada *et al*., 1997).

Iron is the necessary trace elements for human. Although, iron was rich in the nature, it mainly exists in the form of its insoluble salt (Kim *et al*., 2003). Inorganic iron was difficult to be absorbed and do not have biological activities compared with organic iron. The iron mainly exists in the wall of yeast cells and the content of iron in cell wall was almost three times than cell cytoplasm. The cytoplasmic iron was mainly distributed in the mitochondria, vacuole and cytoplasmic sol (Gaudreau *et al*., 2001). For human and animal, trace elements from organic origin, especially from yeast have more advantages (Hegoczki *et al*., 1997). Yeast cells had the ability of enriching trace elements and transforming inorganic iron into organic iron and had a good security and absorbability (Hegoczki, 1994), so it could be used to prevent anemia and other relative diseases. In this experiment, the iron-zymosan could not only provide a highly biological active iron but develop its bioactivity of zymosan itself.

Donzis (1996) reported that zymosan by gavage could improve the content of IL-2 in plasma of mice. Glucan could improve the level of IL-1 and IL-2 in plasma and spleen by injecting through skin, muscle and vein (Sherwood *et al*., 1987).

IL-2 played an important role in lymphocyte development, lymphocytes activation and maintains immune response. It could activate the key cells of the immune system (Waldmann, 1991), IL-2 acted biological effect through the interaction with IL-2 Receptor (IL-2 R) which was on the activated lymphocyte membrane (Romagnani, 1997). The transcript of *IL-2R* gene was another component of the process of T cells activation; it was essential factors for T cells autologous secretes and growth to response of IL-2 (Zhang *et al*., 2001).

The experiment results indicated that iron-zymosan has obvious stimulation for expression of IL-2 and IL-2RmRNA in spleen and had an important regulatory role in cellular immunity and whole immune system in mice.

From 7-14th day, the IL-2mRNA expression in each group except Group V began to increase and from 14-28th day the trend began to decline. The results also indicated that both zymosan and iron-zymosan could promote the express of IL-2mRNA in connection with the growth stage and this effect had certain dose- dependent relation. The iron-zymosan could not only promote expression of IL-2mRNA but keep the content of IL-2 on a higher level on 28th days and thus the biological function was displayed fully.

With the growth time went on, the expression quantity of IL-2R mRNA began to increase from 4-7th days and decline on the 28th day. The expression of IL-2R mRNA began to increase significantly on the 14th day and was dose-dependent to a certain extent. The zymosan had certain stimulation effect on the expression of IL-2R mRNA. The iron in zymosan and zymosan had synergistic effect and could further promote the expression of IL-2R mRNA, prolong the retention time of IL-2R mRNA in the body and had inhibition to the IL-2R mRNA degradation reaction.

The transcription of IL-2mRNA and IL-2RmRNA were significantly increased on 14th day and carried out the immune response. As the transcription of IL-2mRNA and IL-2RmRNA increased, IL-2 and IL-2R could remain at a higher level thus strengthen immune system function, regulate the micro ecological balance in digestive tract, depressed pathogenic bacteria reproduction and enhanced intestinal tract mucosa immune function. On 28th day, the IL-2mRNA and IL-2RmRNA slightly fallen, it indicated that the immune response entered the resting phase, the lymphocytes moved into resting and memory state from the activated state, did not proliferate and differentiate and the volume was smaller (Khan *et al*., 2001).

The digestive tract immune was one part of mucosa immunization and also an important link in the production chain anti-infectious immunity (Bailey and Haverson, 2006). SIgA played an important role and was the main effect factors in digestive tract mucosa immune (Round and Mazmanian, 2009). SIgA was synthetized by IgA^+ cells in proper mucous membrane, firstly, the IgA the epithelial cell was synthetized, then in the process of passing the mucous membrane epidermis, the IgA combine with the fragment secreted by the epithelial cells and finally formed into SIgA (Mestecky and Mcghee, 1987). If the total quantity of IgA^+ cells decreased, the ability of synthesis and secretion of IgA was lowed and also the mucosa immune function was weakened, the intestinal or enterogenous infection arose easily in the end. The changes of SIgA directly reflected the immune status of mucous membrane (Macpherson *et al*., 2001). SIgA could neutralize virus, prevent pathogens adhere to surface epithelial (Giraido *et al*., 2006) and adjust intestinal microbial components and natural antibacterial factor generation. It also had the ability of immune adjustment, immunologic rejection and immune clearance (Fagarasan and Honjo, 2004; Woof and Kerr, 2006; Magalhaes *et al*., 2007).

The results show that the variation of quantity and OD of IgA^+ cells had certain relevance to the dose. The cell number of IgA^+ increased with the dose increasing but between different doses in the same period, the changes of number of cells had no significant difference. The average number of IgA^+ cells increased significantly compared with the control group when the mice were treated continuously for 7 days. The increase was more significant on 14th day. The average number of IgA^+ cells decreased on 28th day but it was still higher than that of the Group V.

SigA could effectively capture pathogens in mucous layer, loss hydrophobicity of the bacteria surface, prevent virus, bacteria and antigen attaching, stimulate sticky protein secretion in intestinal tract mucous membrane surface to degrade virus, promote mobility of mucilage on the surface of mucous membrane and thus expedite excretion of bacteria and endotoxin (Cano and Perdigon, 2003). SIgA was the core substance of intestinal immune barrier (Spahn and Kucharzik, 2004; Fukatsu *et al*., 2006). SIgA could inhibit the reproduction of bacterium and maintain the balance of normal bacteria colony in the intestinal canal (Salminen *et al*., 1996) and played a key role in the course of the local anti-infection (Fuller and Perdigon, 2000).

The composition of cell wall, metabolites and bacteria bodies of probiotics all might stimulate the intestinal mucosa immune systems of bodies (Garriga *et al*., 1998). Iron-zymosan was the main composition of Iron-yeast cell wall and it could promote the generation of SlgA in a short time, improve the content of SlgA in serum and maintain a stable level on 28th day. Both iron-zymosan and zymosan could promote the secretion of SlgA but there was not obvious difference between iron-zymosan and zymosan on 7-28th day. On 14th day, there was larger difference between them but still no significance.

CONCLUSION

Analysing the changes of IgA^+ cells and SIgA content in serum although number of IgA^+ cells showed significantly different in different stage, the content of SlgA in serum hadn't significant differences. It was suggested that there were other regulation mechanism and influence factors in the process of IgA transforming into SIgA (Mestecky and Mcghee, 1987).

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