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Effect of Chitosan on Nitric Oxide Content and Inducible Nitric Oxide Synthase Activity in Serum and Expression of Inducible Nitric Oxide Synthase mRNA in Small Intestine of Broiler Chickens*

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ABSTRACT : The present study was conducted to determine the effects of chitosan on nitric oxide (NO) content and inducible nitric oxide synthase (iNOS) activity in serum, and relative expression of iNOS mRNA in the duodenum, jejunum, and ileum of broiler chickens. A total of 240 one-day-old Arbor Acre mixed-sex broiler chickens were randomly allotted to six dietary treatments with five replicates in each treatment and eight chickens in each replicate. The broiler chickens in the six treatments were fed the basal diet supplemented with 0 (control), 0.05, 0.2, 0.5, 1.0 or 2.0 g/kg chitosan. The trial lasted for 42 days. The results showed that dietary chitosan enhanced NO content and iNOS activity in serum as well as iNOS mRNA expression in the duodenum and ileum of broiler chickens in a quadratic dose-dependent manner ($p<0.05$), and improved jejunum iNOS mRNA expression in a quadratic dose-dependent manner ($p<0.10$) with increasing addition of chitosan. Chicks fed a diet containing 0.5-1.0 g/kg chitosan had higher NO content and iNOS activity in serum as well as small-intestinal iNOS mRNA expression compared with birds given the control diet, but positive effects of chitosan tended to be suppressed when addition of chitosan in the diet was increased to 2.0 g/kg. These results implied that there was a threshold level of chitosan inclusion beyond which progressive reductions in serum NO content and small intestinal iNOS expression occured, and the regulation of chitosan on immune functions in chickens is probably associated with activated expression of iNOS and NO secretion. (**Key Words :** Chitosan, Broiler Chickens, Nitric Oxide, Inducible Nitric Oxide Synthase, mRNA Expression)

INTRODUCTION

Chitosan, a deacetylated chitin, is a natural alkaline polysaccharide with positive charges, and it is widespread in nature. The exoskeletons of arthropods such as crabs, shrimps, insects, and other marine creatures in the crustacean family are good sources of chitosan. In addition, the extraction of chitosan is relatively cheap and easy. Research efforts devoted to elucidating the effects of chitosan on growth and immune functions of animals have been extensive. It had been reported that chitosan could improve growth performance of broiler chickens (Suk, 2004), and it had dose-dependent effects on growth performance in broiler chickens (Shi et al., 2005a), and the higher dietary chitosan had adverse effects on growth in chickens (Razdan and Pettersson, 1996; Razdan et al., 1997). Previous studies have indicated that chitosan could accelerate the wound healing and exert anti-inflammatory effects and stimulate both B and T lymphocytes (Seferian and Martinez, 2000; Chou et al., 2003). Studies in rats demonstrated that chitosan had a significant enhancing effect on cellular and humoral immune function, and activating macrophages for tumoricidal activity and stimulating macrophages to produce interleukin-1 (IL-1) (Nishimura et al., 1986; Zaharoff et al., 2007). Yin et al. (2008) reported that chitosan oligosaccharide could enhance the cell-mediated immune response in early-weaned piglets by modulating the production of cytokines and antibodies. β-1,3/1,6-glucan have a transient immuno-enhancing effect on the cellular and humoral immune function of weanling piglets (Zhong et al., 2008). Shi et al. (2005b) demonstrated that chitosan could strengthen immune functions of broiler chickens.

Nitric oxide (NO) has been shown to be an important factor in immunity, and play a pivotal role in modulating immune responses and inflammation, and it was synthesized from L-arginine by inducible nitric oxide synthase (iNOS) (Moilanen et al., 1999; Korhonen et al.,

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Table 1. Composition and nutritional levels of basal diets (air-dry basis, %)

Items	1-21 d	22-42 d
Ingredients		
Corn	52.68	58.97
Soybean meal	40.00	33.80
Soybean oil	3.00	3.00
Limestone	1.10	1.80
Dicalcium phosphate	1.90	1.25
Sodium chloride	0.37	0.37
Methionine	0.19	0.07
Lysine	0.05	0.03
Choline	0.11	0.11
Mineral additive	0.50	0.50
Vitamin additive	0.10	0.10
Total	100.00	100.00
Nutrient levels		
Metabolic energy (MJ/kg)	12.71	12.78
Crude protein	21.37	18.99
Calcium	1.25	1.10
Available phosphorus	0.62	0.55
Lysine	1.14	1.00
Methionine	0.46	0.34

Supplemental trace elements within a kilogram of basic diet: Fe (as ferrous sulfate), 80 mg; Zn (as zinc sulfate), 80 mg; Mn (as manganese sulfate), 80 mg; Cu (as copper sulfate), 8 mg; I (as calcium iodate), 0.35 mg; Se (as sodium selenite), 0.15 mg.

Supplemental vitamins within a kilogram of basic diet: V_A 3,000 IU, V_D 1,250 IU, V_E 15 IU, V_K 2.2 mg,, V_{B1} 1.5 mg, V_{B2} 8.0 mg, V_{B6} 2.5 mg, V_{B12} 0.011 mg, niacin 44 mg, D-pantothenic acid 11 mg, folic acid 0.9 mg, biotin 0.11 mg, choline 550 mg.

2005; Moncada and Higgs, 2006). The expression of iNOS is essential for the killing of microbes and functions of NO in the regulation of immune responses (Xing and Schat, 2000). It was demonstrated that chitosan could stimulate the macrophages in rats, and increase NO, IL-1 and tumor necrosis factor-α (TNF-α) secretion (Peluso et al., 1994). Porporatto et al. (2003) reported that 0.05% to 0.1% chitosan significantly enhanced the content of NO and expression of iNOS in rat macrophages. This implied that enhanced immune functions in rats by chitosan were associated with increased NO secretion and expression of iNOS.

There is, however, very little data that examines the mechanisms, especially molecular biologic mechanisms, for chitosan to affect immune functions of broiler chickens. It was reported that chicken macrophages and myoblasts could express iNOS (Lin et al., 1996; Shimizu et al., 1998). Therefore, the objective of the present study was to explore the effects of dietary chitosan supplementation on serum NO content and iNOS activity and small intestinal iNOS mRNA expression in broiler chickens and to investigate the probable mechanism for chitosan to affect immune functions of chickens.

MATERIALS AND METHODS

Dietary treatments and management of experimental animals

A total of 240 one-day-old Arbor Acre mixed-sex broiler chickens were randomly allotted to six dietary treatments with five replicates in each treatment, with eight chickens in each replicate. There were no significant differences in initial body weight among treatments ($p>0.05$). The broiler chickens in the six treatments were fed the basal diets supplemented with 0 (control), 0.05, 0.2, 0.5, 1.0 or 2.0 g/kg chitosan. The composition of basal diet is listed in Table 1. Chitosan used in this trial was provided by Jinan Haidebei Marine Bioengineering Limited Company. The degree of deacetylation was determined to be 90.52%, and the particles could pass a 80 mesh screen, and the viscosity was 45 mPa·s. The diets were fed in mash form for 6 weeks. All groups of broiler chickens had free access to feed and water during the whole experimental period. Broiler chickens were maintained on a 23-h light and 1-h dark schedule. The initial room temperature was set at approximately 32°C and reduced by 2 to 3°C weekly until reaching 22°C at wk 4, and then the same temperature was maintained until the end of the trial. All procedures were approved by Animal Care and Use Committee of Inner Mongolia Agricultural University.

Sample collection

At the age of 14, 28 and 42 d, one bird from each replicate of each treatment was randomly selected and blood sample was obtained by cardiac puncture, and centrifuged at 3,000 rpm for 10 min at 4°C to yield serum. Serum was stored at -20°C until analysis of NO content and iNOS activity. Serum NO content was analyzed using commercially available NO kits, and serum iNOS activity was analyzed using iNOS kits (Nanjing Jiancheng Institute of Bioengineering) according to the manufacturer's instructions. The same birds used to obtain blood samples were killed, and duodenum, jejunum and ileum were quickly removed and snap frozen in liquid nitrogen, then stored at -80°C until analysis.

Total RNA isolation and reverse transcription (RT)

Total RNA was isolated from the duodenum, jejunum and ileum using the RNAiso Reagent (TaKaRa, Inc. Dalian, China) according to the manufacturer's instructions. All steps were carried out under RNase-free conditions. RNA integrity was verified electrophoretically by ethidium bromide staining. The RNA purity was determined using UV-clear Microplates (TECAN) at OD260 and an OD260/OD280 ratio. The OD260/OD280 ratio of all samples was >1.80. The RNA yield from the samples was too low to be accurately quantified by spectrometry, so 6.5

Table 2. Effect of different level of chitosan in diet on serum NO content and iNOS activity in chicks

Items	Level of chitosan (g/kg diet)						SEM	p-value	
	0	0.05	0.2	0.5	1.0	2.0		Linear	Quadratic
NO (μmol/L)									
14 d	11.57	14.65	22.37	25.45	33.16	19.28	2.93	0.489	0.008
28 d	8.74	10.22	12.08	14.27	15.42	14.65	2.55	0.088	0.010
42 d	10.03	12.34	12.72	16.20	18.12	8.74	1.69	0.785	0.005
iNOS (U/ml)									
14 d	5.65	11.27	11.05	12.43	11.35	11.20	0.67	0.193	0.071
28 d	11.64	11.79	11.58	14.15	12.01	10.54	1.46	0.453	0.297
42 d	8.33	9.14	9.98	10.35	11.39	9.35	2.25	0.595	0.018

NO = Nitric oxide. iNOS = Inducible nitric oxide synthase.

μl RNA aliquots were amplified. All RNA was treated with RNase-free DNase I to remove any possible genomic DNA contamination. For amplification of the targets, RT and PCR were run in two separate steps. Total RNA were reverse transcribed with PrimeScript™ RT Reagent Kit (TaKaRa, Inc. Dalian, China) following the manufacturer's directions and performed by 37°C for 15 min for reverse transcription, 85°C for 5 sec to inactivate the reverse transcriptase. The RT reaction mixture (10 μl) contained 0.5 μl PrimeScript™ RT Enzyme Mix I, 2 μl 5×PrimeScript Buffer, 0.5 μl Random 6 mers and 0.5 μl Oligo dT Primer. The RT products (cDNA) were stored at -20°C pending quantitative PCR assay.

Real-time PCR for quantification of iNOS mRNA

Primers used in this study were as follows: β-Actin (118 bp, GenBank accession no. NM_205518) 5'-GCCAACAGA GAGAAGATGACAC-3' (forward) and 5'-GTAACACCAT CACCAGAGTCCA-3' (reverse); iNOS (371bp, GenBank accession no. U46504) 5-AGGCCAAACATCCTGGAGG TC-3 (forward) and 5-TCATAGAGACGCTGCTGCCAG-3 (reverse). The thresh-old cycle (Ct) value represents the cycle number at which sample fluorescence rises statistically above back-ground. Relative levels of iNOS mRNA were quantified using SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Inc. Dalian, China) following manufacturer's instructions and a DNA Engine Opticon2 fluorescence detection system (MJ research, USA) according to optimized PCR protocols. Reactions were also performed with negative controls (water replacing cDNA). The PCR reaction system (20 μl) contained 10 μl 2×SYBR® Premix Ex Taq™ and 0.4 μl (10 μM) each of forward and reverse specific primers, 2 μl of cDNA template and 7.2 μl RNA Free H_2O. The same dilution was used for both iNOS and β-actin. For the PCR reaction, the following experimental run protocol was used: denaturation program (95°C for 1 min), amplification and quantification program repeated 45 times (5 sec at 95°C for denaturation, 30 sec at 62°C for annealing, and 10 sec at 72°C for extension), melting curve program (70 to 95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement). Fluorescence data was acquired after the extension step during PCR reactions that contained SYBR Green. Thereafter, PCR products were analyzed by generating a melting curve. The melting curve of a product is sequence-specific and can be used to distinguish non-specific from specific PCR products. Real time PCR efficiencies of iNOS and β-actin were acquired by amplification of a dilution series of PCR products and were close to one. Gene expression was presented using a modification of the $2^{-\Delta Ct}$ method, first described by Livak in PE Biosystems Sequence Detector User Bulletin 2 (Livak and Schmittgen, 2001). Expression levels of iNOS was calculated as relative values using the $2^{-\Delta Ct}$ method, where ΔCt was equal to Ct of the iNOS minus Ct of the β-actin (Tropea et al., 2007). The sizes of RT-PCR products were confirmed by gel electrophoresis on 2% agarose gels stained with ethidium bromide, and bands were visualized by exposure to ultraviolet light. Sequences were confirmed by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Statistical analysis

All data were subjected to General Linear Model procedure of SAS software (SAS Institute, 1998). Regression analysis was conducted to evaluate linear and quadratic effects of chitosan on the various response criteria. A level of $p<0.05$ was used as the criterion for statistical significance while level of 0.10 was taken to indicate a statistical trend.

RESULTS

Serum NO content and iNOS activity

As shown in Table 2, broiler chicks fed the chitosan diets had higher NO concentrations and iNOS activities in serum than those fed the control diets, and the content of serum NO increased quadratically with increasing addition of chitosan during the whole experimental period ($p<0.05$). Serum iNOS activity also increased quadratically with increasing addition of chitosan on day 42 ($p<0.05$). On day

Table 3. Effect of different level of chitosan in diet on relative expression of iNOS mRNA in small intestine of chicks ($2^{-\Delta Ct}$)

Items	Level of chitosan (g/kg diet)						SEM	p-value	
	0	0.05	0.2	0.5	1.0	2.0		Linear	Quadratic
Duodenum									
14 d	0.0096	0.0153	0.0152	0.0169	0.0197	0.0151	0.003	0.467	0.119
28 d	0.0380	0.0427	0.0575	0.0595	0.0641	0.0510	0.011	0.483	0.059
42 d	0.0168	0.0174	0.0190	0.0270	0.0286	0.0266	0.006	0.090	0.010
Jejunum									
14 d	0.0053	0.0078	0.0089	0.0091	0.0147	0.0089	0.001	0.113	0.003
28 d	0.0295	0.0294	0.0320	0.0337	0.0449	0.0314	0.010	0.558	0.072
42 d	0.0126	0.0179	0.0196	0.0235	0.0237	0.0220	0.003	0.209	0.083
Ileum									
14 d	0.0116	0.0144	0.0135	0.0153	0.0156	0.0143	0.003	0.391	0.189
28 d	0.0204	0.0240	0.0235	0.0261	0.0244	0.0175	0.005	0.259	0.054
42 d	0.0104	0.0159	0.0177	0.0276	0.0307	0.0198	0.005	0.407	0.008

iNOS = Inducible nitric oxide synthase.

14, 28 and 42, broiler chickens fed the diets containing 0.5 to 1.0 g/kg chitosan had the higher NO concentrations and iNOS activities in serum than those fed other diets, whereas NO concentrations and iNOS activities in serum tended to decrease after the addition of chitosan in diets increased to 2.0 g/kg.

Relative expression of iNOS mRNA

As shown in Table 3, compared with the control, feeding chitosan enhanced iNOS mRNA expression in duodenum, jejunum, and ileum of broiler chickens over the whole experimental period, except for that in ileum at the age of 28 d. According to regression analysis, iNOS mRNA expression in duodenum and ileum tended to increase ($p<0.10$) or increased quadratically ($p<0.05$) with increasing addition of chitosan on day 28 and 42, and that in jejunum also increased quadratically with increasing addition of chitosan on day 14 ($p<0.05$). Broiler chicks fed with the diet containing 0.5 to 1.0 g/kg chitosan had greater iNOS mRNA expression in duodenum, jejunum and ileum over the whole experimental period than other ones. However, progressive iNOS mRNA expression reductions occurred when chitosan was increased to 2.0 g/kg diet.

DISCUSSION

There are studies showing that NO, a free radical generated from L-arginine by iNOS, is an important chemical in numerous physiological processes (e.g., as a neurotransmitter and vasodilator) (Moncada et al., 1991; Bredt and Snyder, 1992). In addition, NO is recognized as an important factor in nonspecific immunity with microbiocidal activities against a broad spectrum of protozoa, fungi, bacteria, and viruses (Granger et al., 1988; James and Glavin, 1989; Liew et al., 1990; Alspaugh and Granger, 1991; Denis, 1991; Nathan and Hibbs, 1991). NO is also a highly reactive signaling molecule and inflammatory mediator, which acts as a cytotoxic agent and modulates immune responses and inflammation (Moilanen et al., 1999; Korhonen et al., 2005).

There are many evidences that polysaccharides may act as immune enhancers or immunomodulators, and affect NO production, especially macrophage NO secretion, thereby modulate immune function. For example, Glycyrrhiza polysaccharides induced NO production by murine peritoneal macrophages *in vitro* (Nose et al., 1998). Lentinan and polyporus polysaccharide increased the production of NO by mouse peritoneal macrophages in a concentration-depend manner and showed a cooperative effect with interferon-γ (IFN-γ), and the effect was blocked effectively by a nitric oxide synthase (NOS) inhibitor (Huang et al., 1999; Hou et al., 2000). Chitosan is a linear polymer of N-acetyl-D-glucosamine and deacetylated glucosamine, and Peluso et al. (1994) demonstrated that it could stimulate the macrophages in rats and increase NO secretion. Yu et al. (2004) indicated that oligochitosan could significantly increase the activity of iNOS and induce the synthesis of NO in macrophages. Deng et al. (2008) also reported that dietary supplementation of chito-oligosaccharide appeared to improve the immunity of broilers by promoting the weight of the main immune organs, increasing immunoglobulin M (IgM) secretion, and stimulating microphages to release TNF-α, IL-1, interleukin-6 (IL-6) and IFN-β, and activating iNOS to induce NO. The present study showed a dose-dependent relationship between supplemental dosage of chitosan in diets and serum content of NO in broiler chickens. The dietary chitosan quatratically improved serum NO content, and the addion of 0.5 to 1.0 g/kg chitosan in diets had better effects. But after the addition of chitosan in diet was increased to 2.0 g/kg, beneficial effects of chitosan on NO content in serum tended to be suppressed. In addition, NO was generated from L-arginine by iNOS (Moncada and Higgs, 2006), and Zhang et al. (2006) demonstrated that

NO production and iNOS activity in the spleen of broiler chicks were correlated. In our study serum NO contents also tended to increase with increasing serum iNOS activity in most cases, but there existed the contradiction about it in some cases. The causes of this result are unclear, and the probable reason is that serum NO content and iNOS activity were influenced by more other factors than NO production and iNOS activity in tissues. Therefore, more researches are required to confirm and interpret the phenomenon.

iNOS is responsible for most NO production (Morris and Billiar, 1994), and its expression is essential for the killing of microbes and functions of NO in the regulation of immune responses (Xing and Schat, 2000). Wang et al. (1999) explored the mechanisms of protein-bound polysaccharide (PSK) increased NO secretion of macrophages, and showed that PSK could induced iNOS mRNA and protein expression. Porporatto et al. (2003) demonstrated that the expression of iNOS in rat macrophages could be influenced by chitosan. However, little is known about the effects of chitosan on iNOS activity and expression in poultry. Chicken macrophages and myoblasts can express iNOS (Lin et al., 1996; Shimizu et al., 1998). Both of these two kinds of cells are contained in small intestinal tissues, thus they may be the sources of iNOS in small intestine. In the present study, it was shown that the diets containing 0.5 and 1.0 g/kg chitosan improved serum iNOS activity and enhanced iNOS expression in duodenum, jejunum, and ileum of broiler chickens. Therefore, enhanced iNOS activity and expression may be one of several mechanisms by which chitosan increases the production of NO and stimulates the immune functions of chickens. Chitosan may have a future potential to be used as feed additives in animal feed because of its non-toxicity, biodegradability and biocompatibility, low side effects, promoting the growth performance and improving immune functions. The results of the current work also indicated that iNOS mRNA expressions in all duodenum, jejunum and ileum were higher at 28 d than at others. Little is known about small intestinal iNOS mRNA expressions at different ages of broiler chicks. Therefore, further studies are needed to investigate and interpret the phenomenon.

Our present study indicated that dietary chitosan tended to decrease serum NO content and iNOS activity and lessen iNOS expression at a higher level of inclusion (2.0 g/kg diet). This suggested that there was a threshold level of chitosan inclusion beyond which progressive iNOS expression reductions in broiler chickens occured, and at 0.5 to 1.0 g/kg level of inclusion the best effects were attained with chitosan. The causes about this are unclear. Our previous study indicated 0.5 g/kg chitosan supplementation in diet was optimal for broiler growth and antibody titer of Newcastle disease, but 1.0 to 5.0 g/kg chitosan supplementation in diet tended to enhance lymphocyte proliferation (Shi et al., 2005b). Therefore, further work is needed to confirm the mechanism by which different levels of chitosan affects immune functions in broiler chickens.

IMPLICATIONS

Our results indicated that the influence of chitosan on NO content and iNOS activity in serum as well as iNOS mRNA expression in small intestine of broiler chickens represented a quadratic relationship, and feeding 0.5 to 1.0 g/kg chitosan showed the best enhanced effect, while feeding 2.0 g/kg chitosan showed the progressive reductions. This implied that there was a threshold level of chitosan inclusion beyond which progressive reductions in serum NO content and small intestinal iNOS expression occured, and the mechanism by which chitosan modulated immune functions in chickens were probably that chitosan activated expression of iNOS and NO secretion.

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