doi: 10.17221/8405-CJAS

Effects of chitosan on nitric oxide production and inducible nitric oxide synthase activity and mRNA expression in weaned piglets

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ABSTRACT: The effects of chitosan on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) activity and gene expression *in vivo* or *vitro* were investigated in weaned piglets. *In vivo*, 180 weaned piglets were assigned to five dietary treatments with six replicates. The piglets were fed on a basal diet supplemented with 0 (control), 100, 500, 1000, and 2000 mg chitosan/kg feed, respectively. *In vitro*, the peripheral blood mononuclear cells (PBMCs) from a weaned piglet were cultured respectively with 0 (control), 40, 80, 160, and 320 μg chitosan/ml medium. Results showed that serum NO concentrations on days 14 and 28 and iNOS activity on day 28 were quadratically improved with increasing chitosan dose ($P < 0.05$). The iNOS mRNA expressions were linearly or quadratically enhanced in the duodenum on day 28, and were improved quadratically in the jejunum on days 14 and 28 and in the ileum on day 28 ($P < 0.01$). *In vitro,* the NO concentrations, iNOS activity, and mRNA expression in unstimulated PBMCs were quadratically enhanced by chitosan, but the improvement of NO concentrations and iNOS activity by chitosan were markedly inhibited by *N*-(3-[aminomethyl] benzyl) acetamidine (1400w) ($P < 0.05$). Moreover, the increase of NO concentrations, iNOS activity, and mRNA expression in PBMCs induced by lipopolysaccharide (LPS) were suppressed significantly by chitosan ($P < 0.05$). The results indicated that the NO concentrations, iNOS activity, and mRNA expression in piglets were increased by feeding chitosan in a dose-dependent manner. In addition, chitosan improved the NO production in unstimulated PBMCs but inhibited its production in LPS-induced cells, which exerted bidirectional regulatory effects on the NO production via modulated iNOS activity and mRNA expression.

Keywords: serum; mononuclear cell; small intestine; immune function; lipopolysaccharide

INTRODUCTION

To improve the production efficiency, piglets are often weaned early in modern swine production. However, weaning has been shown to be stressful and could impair or affect the development of immune function in piglets, leading to their higher liability to infection by pathogens (Pie et al. 2004; Johnson et al. 2006). In the past decades, antibiotics have frequently been used in swine diets, particularly in the diets of newly weaned pigs, for the prophylaxis of infections (Bosi et al. 2011). However, this practice has major consequences, such as acquired drug resistance in animals and humans, and residual antibiotics in animal products (Geerts and Gryseels 2000; Mary 2000). Thus, it is extremely urgent to develop alternative dietary additives to improve the immune function of weaned piglets.

Chitosan, a natural and nontoxic alkaline polysaccharide, is formed by the action of chitin deacetylases and is a key structural component of helminths, arthropods, and fungi (Synowiecki and Al-Khateeb 2003). Chitosan has been shown to be an ideal additive to promote systemic and mucosal immune functions of animals. Yin et al. (2008) indicated that chitosan might enhance the

Supported by the National Natural Science Foundation of China (Project No. 31060310).

The authors have no conflicts of interest in this study.

doi: 10.17221/8405-CJAS

cell-mediated immune response in early-weaned piglets by modulating the production of cytokines and antibodies. Porporatto et al. (2005) found that the uptake of chitosan involved the antigen-presenting cells in the intestinal of rats after oral administration, and this mucoadhesive polysaccharide profoundly affected intestinal immunity by enhancing the T helper cell type 2 (Th2)/Th3 microenvironment in the mucosa. Li et al. (2009) found that dietary supplementation with chitosan enhanced immune functions which were associated with the increase of nitric oxide (NO) secretion and inducible nitric oxide synthase (iNOS) mRNA expression in the small intestine of broilers. So far, however, there have been few studies conducted to evaluate the effect of chitosan on innate immune function in piglets involved the NO pathway.

NO is an important host defence effector in innate immunity. It is generated in various tissues from L-arginine by different forms of nitric oxide synthase: neuronal NOS, iNOS, and endothelial NOS (Nathan and Stuehr 1990). As a toxic defense molecule against infectious organisms, NO regulates the functional activity, growth, and death of many immune and inflammatory cells, including macrophages, T lymphocytes, antigen presenting cells, mast cells, neutrophils, and natural killer cells (Tripathi et al. 2007). Inducible NOS, which was originally discovered in activated macrophages and predominantly functions in the innate immune system, is expressed in dendritic cells, NK cells, monocytes, macrophages among other immune cells (Bogdan 2001; David et al. 2012). When iNOS is induced, NO will be produced and its proper expression is essential for improving the functions of NO in the regulation of immune responses (Xing and Schat 2000). However, also excessive NO production is known to induce tissue damage.

Results of the *in vitro* trial showed that chitosan significantly enhanced the NO content and iNOS expression in rat macrophages (Porporatto et al. 2003). However, Yang et al. (2010) indicated that chitosan markedly inhibited the LPS-induced NO production of RAW 264.7 cells. These investigations involving the effects of chitosan on the iNOS-derived NO mainly were focused on macrophages but not peripheral blood mononuclear cells (PBMCs) and their conclusions were not coincident. PBMCs, which play an important role in disease monitoring, inflammation response, and the removal of pathogens, comprise several cell types such as CD4+ and CD8+ T lymphocytes, B lymphocytes, natural killer cells, monocyte/macrophages, and dendritic cells and were a more comprehensive reflection of the immunocyte component than macrophages (Mesko et al. 2011; Liu et al. 2012). In addition, PBMCs are easy to be acquired and analyzed, inexpensive to be processed. Therefore, in the present trial *in vitro*, the effects of chitosan on the iNOS-derived NO in piglet PBMCs were studied.

The objective of the present study was to explore the effects of chitosan on the NO concentrations, iNOS activity, and mRNA expression of piglets *in vivo* and *in vitro*. The findings of this study may provide useful evidence for the application of chitosan as a dietary supplement to promote the immune functions and health of weaned piglets.

MATERIAL AND METHODS

All procedures described in this study were approved by the Animal Care and Use Committee of the Inner Mongolia Agricultural University.

In vivo trial design. This study was designed as single factor randomized block arrangement, with five dietary treatment groups supplemented

Table 1. Composition and nutrient levels of the basal diet (air dry basis, %)

Ingredients	Content	Nutrients	Level
Corn	51.90	digestible energy (MJ/kg)	14.32
Soybean meal	16.00	crude protein	20.02
Wheat	20.00	crude fat	3.0
Fish meal	2.50	crude fibre	4.2
Corn gluten meal	2.00	calcium	0.72
Whey powder	2.00	phosphorus	0.56
Soya bean oil	2.00	lysine	1.35
Limestone	0.70	methionine + cystine	0.82
$CaHPO_4$	1.00	threonine	0.74
NaCl	0.30		
Premix[1]	1.60		
Total	100		

[1]premix provides the following nutrients per kg diet: vitamins: A 16 000 IU, D3 2500 IU, E 60 IU, K3 4.5 mg, B1 2.6 mg, B2 8.7 mg, B6 7.0 mg, B12 0.03 mg, C 200 mg, pantothenic acid 13 mg, nicotinic acid 35 mg, biotin 0.47 mg, folic acid 0.85 mg, iron 155 mg, copper 35 mg, zinc 100 mg, manganese 25 mg, iodine 0.35 mg, cobalt 0.2 mg, selenium 0.25 mg, choline chloride 750 mg, phytase 500 FTU, lysine 6.20 g, methionine 2.20 g, threonine 1.10 g

doi: 10.17221/8405-CJAS

respectively with 0 (control), 100, 500, 1000 or 2000 mg chitosan/kg feed. The formation of trial diets followed NRC (1998) standards (Table 1). All diets were offered in meal form. Chitosan used in this trial was provided by Jinan Haidebei Marine Bioengineering Co. Ltd. (Jinan, China). The deacetylation degree of chitosan was determined to be 85.09% and the viscosity was 45 cps.

Animals and management. A total of 180 piglets (Duroc × Large White × Landrace), which were obtained from a local commercial farm, were assigned randomly into five treatment groups with six repetitions (three pens of females and three pens of males) in each treatment, and six piglets in each replicate/pen (2.0 × 2.2 m). Piglets were weaned at 28 days of age, with an initial average body weight of 7.6 kg, and penned in a temperature-controlled nursery building, where the temperature was maintained at 26–28°C and relative humidity was about 65–70%. The weaned piglets had 1 week of housing and management adaptation before the trial phase. The trial period was 28 days. Feed and water were freely available during the trial period.

Sample collection. On days 14 and 28 of the trial, one pig from each replicate of each treatment was randomly selected and blood samples were obtained by puncturing the *vena cava*. The blood samples were centrifuged at 1200 *g* for 10 min at 4°C to obtain serum. Serum was stored at –20°C until analysis of NO content and iNOS activity.

For determining the expression of iNOS mRNA, the pigs used to obtain blood samples were sacrificed, and the duodenum, jejunum, and ileum were quickly removed and frozen in liquid nitrogen, and then stored at –80°C until analysis.

Isolation of PBMCs. PBMCs from a healthy weaned piglet (Duroc × Large White × Landrace) were isolated using lymphocyte separation medium (TBD, Tianjin, China) according to the manufacturer's instructions. The blood samples were centrifuged (800 *g*, at 20°C for 20 min), and the lymphocyte layer was recovered and washed twice with RPMI-1640 medium (Gibco, USA). Subsequently, the cells were counted using a hemocytometer and a light microscope by a trypan blue exclusion method, and resuspended using an adequate volume of complete RPMI-1640 medium containing 10% (v:v) heat-inactivated fetal calf serum, 100 U/l of penicillin, 100 mg/l of streptomycin, and 25 mmol/l HEPES buffer (all Sigma Aldrich, St. Louis, USA).

In vitro trial design and cell culture. Three *in vitro* trials were designed to study the effect of chitosan on iNOS-derived NO in piglet PBMCs. In trial 1, the effects of different chitosan levels on NO production were investigated. This trial was designed as five treatment groups with six replicates in each treatment. Cell suspensions (1×10^6 cells/l of culture medium) were allocated into a 24-well plate with a total volume of 1 ml culture medium per well which contained chitosan at final concentrations of 0, 40, 80, 160, and 320 μg/ml respectively, and cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. At the end of a 24-hour incubation, supernatants were collected after centrifugation (800 *g* at 4°C for 10 min) and stored at –20°C for analysis of NO and iNOS. The cells were harvested and stored at –80°C until analyzed for mRNA expression of iNOS.

In trial 2, the *N*-(3-[aminomethyl] benzyl) acetamidine (dihydrochloride, 1400 w) (Beyotime Institute of Biotechnology, Haimen, China), a highly selective iNOS inhibitor, was used to confirm that NO was generated from iNOS. Three treatment groups were control (without chitosan and 1400w), chitosan (80 μg/ml), chitosan (80 μg/ml) + 1400w (1mM) with six replicates in each treatment. The adding dosage of chitosan was set to be 80 μg/ml medium, according to the results of trial 1. The process of cell culture and treatment was the same as in trial 1.

In trial 3, the effects of chitosan on the NO production, iNOS activity, and mRNA expression of PBMCs induced by LPS (LPS from *Escherichia coli* serotype 055:B5; Sigma Aldrich) were studied. The four treatment groups were control (without chitosan and LPS), LPS (1 μg/ml), LPS (1 μg/ml) + chitosan (80 μg/ml), LPS (1 μg/ml) + chitosan (160 μg/ml) with six replicates in each treatment. Chitosan and LPS were added simultaneously to each well except control group at the beginning of trial. The adding concentration of chitosan was respectively 80 and 160 μg/ml according to the results from trial 1. The process of cell culture and treatment was similar to that in trial 1.

Detection of NO content and iNOS activity. NO content was determined as described by Shi (2004). Briefly, 100 μl serum or supernatant was mixed with 100 μl Griess reagent (1% sulfanilamide and 0.1% *N*-naphthylethyl-ethylenediamine dihydrochloride in 5% phosphoric acid), and then the absorbance at 540 nm was measured with an enzyme-linked immunoabsorbent assay plate reader,

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and nitrite concentration was determined using a curve calibrated on sodium nitrite standards. The iNOS activity in the serum and culture fluid was analyzed using commercial iNOS kits (Nanjing Jiancheng Institute of Bioengineering, China) according to the manufacturer's instructions. One activity unit of iNOS was defined as the production of 1 nmol NO per ml of serum or supernatant per min.

Total RNA isolation and reverse transcription (RT). For determining iNOS gene expression by quantitative reverse-transcribed PCR, RNA was extracted from the duodenum, jejunum, ileum, and PBMCs of pigs using the RNAiso Reagent (TaKaRa Inc., Dalian, China) according to the manufacturer's instructions. RNA integrity was verified electrophoretically by ethidium bromide staining and the RNA purity was assayed by the OD_{260}/OD_{280} ratio using UV-clear microplates (TECAN, Männedorf, Switzerland) at OD_{260} and OD_{280}. 2 µl of total RNA of each sample were added to the RT reaction mixture (10 µl), which contained 0.5 µl PrimeScript™ RT Enzyme Mix I, 2 µl 5× PrimeScript Buffer, 0.5 µl Random 6 mers, 0.5 µl Oligo dT Primer and free RNase water, for the formation of cDNA using PrimeScript RT Reagent Kit (TaKaRa Inc.) following the manufacturer's directions. The RT reaction parameters were as follows: RT at 37°C for 15 min, and RT inactivation at 85°C for 5 s. The RT products (cDNA) were stored at –20°C for quantitative PCR assay.

Relative quantification of real-time PCR for iNOS. The primers were designed specifically using an online primer design tool (http://www.ncbi.nlm.nih.gov/tools /primer-blast/index) according to gene sequences in GenBank (http://www.ncbi.nlm.nih.gov; β-actin – XM_003124280.2; iNOS – NM_001143690.1), where Sus scrofa β-actin was used as the housekeeping gene: primer for Sus scrofa β-actin (forward 5'- TACAC CGCTA CCAGT TCGCC AT-3'; reverse 5'-TCTCC ATGTC GTCCC AGTTG GT-3'; 270 bp) and Sus scrofa iNOS (forward 5'-AAACA CACAA GCTGG CCTCC CTT-3'; reverse 5'-TGCAT GAGCA CAGCG GCAAA-3'; 385 bp) were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Relative levels of iNOS mRNA were quantified using SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Inc.) following the manufacturer's instructions. Reactions were also performed with negative controls (using water instead of cDNA). The PCR reaction system (20 µl) contained 10 µl 2× SYBR® Premix Ex Taq™ and 0.4 µl (10µM) of forward and reverse specific primers, 2 µl of cDNA template, and 7.2 µl RNA-free H_2O. The following procedure was used for amplification: one cycle at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C for denaturation, 30 s at 60°C for annealing, and 30 s at 72°C for extension; and melting curves were determined between 70 and 95°C with a heating rate of 0.5°C/s and continuous fluorescence measurement. Fluorescence data were acquired after the extension step during PCR reactions that contained SYBR Green. Expression levels of iNOS were calculated as relative values using the $2^{-\Delta\Delta Ct}$ method (Kenneth and Thomas 2001). The sizes of RT-PCR products were confirmed by 2% agarose gel electrophoresis in the presence of ethidium bromide, and bands were visualized by exposure to ultraviolet light. Sequences were confirmed by the Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Statistical analysis. Regression analysis was conducted to evaluate linear and quadratic effects of chitosan on the various response criteria in piglets using SAS software (Statistical Analysis System, Version 9.0, 2003) Data in *in vitro* trials referred to the LPS and 1400w were analyzed by ANOVA followed by Fisher's Least Significant Difference test. $P < 0.05$ was considered statistically significant.

RESULTS

NO concentration and iNOS activity in serum. With the increasing addition of chitosan, NO concentrations improved in a significant quadratic dose-dependent manner ($P < 0.05$), which were higher when the diets contained 500–1000 mg/kg chitosan on day 14 or 28 (Table 2). Similar to the change in NO concentrations, iNOS activity also increased quadratically ($P < 0.05$) and reached a maximum value of 25.2 U/ml when the diet was supplemented with 500 mg/kg chitosan on day 28. However, both NO concentration and iNOS activity were reduced when chitosan supplementation was increased to 2000 mg/kg.

Relative expression of iNOS mRNA in pig small intestine. Compared with the control, iNOS mRNA expressions in the chitosan treatments were increased (Table 3). With increasing chitosan, the iNOS mRNA expressions were enhanced linearly

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Table 2. Effects of chitosan on NO content and iNOS activity in serum of weaned piglets

Items	Level of chitosan (mg/kg)					SEM	*P*-value	
	0	100	500	1000	2000		linear	quadratic
NO (μmol/l)								
Day 14	33.46	39.01	42.10	43.33	38.70	2.760	0.391	0.040
Day 28	43.64	47.78	53.52	49.63	44.07	2.492	0.643	0.016
iNOS (U/ml)								
Day 14	18.41	22.30	22.89	23.48	21.15	1.239	0.722	0.075
Day 28	21.99	23.63	25.20	24.75	22.04	1.029	0.702	0.027

NO = nitric oxide, iNOS = inducible nitric oxide synthase, SEM = standard error of the mean
$P < 0.05$ (significant regression relation), $P < 0.01$ (extremely significant regression relation)

Table 3. Effects of chitosan on iNOS mRNA relative expression in small intestines of weaned piglets ($2^{-\Delta\Delta Ct}$)

Items	Level of chitosan (mg/kg)					SEM	*P*-value	
	0	100	500	1000	2000		linear	quadratic
Duodenum								
Day 14	1.115	1.464	1.618	1.691	1.405	0.180	0.495	0.076
Day 28	1.022	2.098	2.563	3.984	2.392	0.411	0.016	< 0.0001
Jejunum								
Day 14	1.090	1.288	1.499	1.899	1.306	0.164	0.294	0.0001
Day 28	1.097	1.100	1.170	2.314	0.997	0.253	0.644	0.001
Ileum								
Day 14	1.045	1.295	1.610	1.435	1.355	0.154	0.375	0.099
Day 28	1.045	2.245	2.291	2.476	1.131	0.318	0.338	0.0001

NO = nitric oxide, iNOS = inducible nitric oxide synthase, SEM = standard error of the mean
$P < 0.05$ (significant regression relation), $P < 0.01$ (extremely significant regression relation)

($P < 0.05$) or quadratically ($P < 0.01$) in the duodenum on day 28, and improved quadratically in the jejunum on days 14 and 28 and in the ileum on day 28 ($P < 0.01$). In addition, 500 and 1000 mg/kg chitosan treatments had higher iNOS mRNA expressions in the duodenum, jejunum, and ileum compared with other treatments. However, the positive effects of chitosan tended to be suppressed when the additional dose of chitosan was increased to 2000 mg/kg.

NO concentration, iNOS activity, and mRNA relative expression in PBMCs. To study the effect of different levels of chitosan on the NO production in PBMCs of weaned pigs, the NO concentration, iNOS activity, and mRNA expression were measured (Table 4). The results showed that, with increasing chitosan, NO concentration and iNOS activity and mRNA expression were improved quadratically ($P < 0.05$). However, the NO concentrations, iNOS activities, and mRNA expression were decreased when supplemented with 320 μg/ml chitosan in the culture medium.

NO concentration, iNOS activity, and mRNA relative expression in PBMCs in the presence of 1400w. To confirm that NO was generated from iNOS, 1400w, a highly selective iNOS inhibitor, was used in *in vitro* trial 2 (Table 5). The NO production and iNOS activities in chitosan (80 μg/ml) + 1400w (1mM) group were inhibited significantly ($P < 0.05$) by 1400w compared with chitosan (80 μg/ml) group, which suggested that chitosan improves the NO production via iNOS.

NO concentration, iNOS activity, and mRNA relative expression in PBMCs induced by LPS. In *in vitro* trial 3, there was significant increase of NO concentration, iNOS activity, and mRNA expression induced by LPS compared with control group (Table 6), however, the supplementation of 80 or 160 μg/ml chitosan inhibited significantly ($P < 0.05$) the increase by LPS.

doi: 10.17221/8405-CJAS

Table 4. Effects of chitosan on NO content, iNOS activity, and iNOS mRNA relative expression ($2^{-\Delta\Delta Ct}$) in peripheral blood mononuclear cells

Items	Level of chitosan (μg/ml)					SEM	*P*-value	
	0	40	80	160	320		linear	quadratic
NO (μmol/l)	24.65	26.42	31.34	27.4	25.14	1.141	0.527	0.001
iNOS (U/ml)	12.78	13.61	19.23	15.67	14.56	1.332	0.435	0.040
iNOS mRNA	1.03	1.31	1.70	1.63	1.47	0.177	0.160	0.017

NO = nitric oxide, iNOS = inducible nitric oxide synthase, SEM = standard error of the mean
$P < 0.05$ (significant regression relation), $P < 0.01$ (extremely significant regression relation)

Table 5. Effects of chitosan on NO content, iNOS activity, and iNOS mRNA relative expression ($2^{-\Delta\Delta Ct}$) in peripheral blood mononuclear cells in the absence or presence of 1400w

Items	Control	COS	COS+1400w
NO (μmol/l)	24.29 ± 3.07^{b}	30.79 ± 3.98^{a}	25.71 ± 4.35^{b}
iNOS (U/ml)	13.28 ± 1.67^{b}	20.03 ± 2.50^{a}	15.32 ± 3.14^{b}
iNOS mRNA	1.06 ± 0.35^{b}	1.85 ± 0.41^{a}	1.83 ± 0.29^{a}

COS = chitosan (80 μg/ml), NO = nitric oxide, iNOS = inducible nitric oxide synthase, 1400w = highly selective iNOS inhibitor
^{a,b}in the same row, means with the same superscript are not signicantly different ($P > 0.05$), with adjacent superscripts are signicantly different ($P < 0.05$)

DISCUSSION

During the weaning period, piglets are very sensitive to infection and the mortality and morbidity of the herd are higher in comparison to other periods. Therefore, feed additives that boost the immune responses of weaned piglets are highly recommended. It is reported that chitosan modulated innate immune function in broilers, and this may be related to the NO production (Deng et al. 2008; Li et al. 2009). NO is an endogenously produced molecule that plays an integral role in defending against bacteria, fungi, viruses, and parasites in innate immune response, which are involved in immune cell signalling pathway and the biochemical reactions (David et al. 2012).

In our study, the NO concentrations as well as iNOS activity in the serum and the mRNA expression in small intestine of piglets were improved in a dose-dependent manner with increasing chitosan supplementation. Shi (2004) showed that dietary supplementation with chitosan enhanced NO content and iNOS activity in the serum of broilers. Li et al. (2009) found that the NO content and iNOS activity in the serum and iNOS mRNA expression in the small intestine of broilers were improved by feeding chitosan. The findings of the current research were consistent with those of previous studies. It was reported that the strong stimulatory activity of chitosan has been mainly attributed to the *N*-acetyl-D-glucosamine residues and its effect could be mediated by combined action with a macrophage mannose specific receptor (Porporatto et al. 2003; Feng et al. 2004). Zhao (2004) indicated that macrophages activated by oligochitosan in-

Table 6. Effects of chitosan on NO content, iNOS activity, and iNOS mRNA relative expression ($2^{-\Delta\Delta Ct}$) in peripheral blood mononuclear cells treated by LPS

Items	Control	LPS	COS A + LPS	COS B + LPS
NO (μmol/l)	24.44 ± 4.49^{c}	67.38 ± 3.91^{a}	57.00 ± 3.37^{b}	52.67 ± 6.29^{b}
iNOS (U/ml)	13.86 ± 2.07^{c}	26.98 ± 3.00^{a}	21.27 ± 4.40^{b}	19.56 ± 1.91^{b}
iNOS mRNA	1.06 ± 0.40^{c}	3.09 ± 0.48^{a}	2.47 ± 0.14^{b}	2.15 ± 0.33^{b}

LPS = lipopolysaccharide, COS A = 80 μg chitosan per ml medium, COS B = 160 μg chitosan per ml medium, NO = nitric oxide, iNOS = inducible nitric oxide synthase, 1400w = highly selective iNOS inhibitor
$^{a–c}$in the same row, means with the same superscript are not signicantly different ($P > 0.05$), with adjacent superscripts are signicantly different ($P < 0.05$)

doi: 10.17221/8405-CJAS

duced iNOS gene expression and improved the activity of iNOS and NO production. Porporatto et al. (2005) found that chitosan could be absorbed by the antigen-presenting cells and profoundly affected immunity by activating the T cell in the intestinal mucosa of rats. Although its mechanism of action is not entirely clear, the NO production in this study may be associated with increase of iNOS activity in the serum and iNOS mRNA expression in the small intestine of piglet by chitosan. Moreover, NO mediates multiple physiological functions in the gastrointestinal tract, including mucosal blood flow, maintenance of mucosal integrity, and maintenance of vascular tone (Angel 2008). So, we concluded that proper production of NO by chitosan may be positive for improving the immune and intestinal mucosal function. Our previous study indicated that chitosan could quadratically improve growth in weaned pigs, which may be a reflection of this positive effect (Xu et al. 2013).

In the present study, three *in vitro* trials were designed in order to further investigate the effects of chitosan on NO production and iNOS activity and gene expression in piglet PBMCs. It was found that chitosan increased NO concentration and iNOS activity in the culture fluid and iNOS mRNA expression in the unstimulating PBMCs in a quadratic manner. However, increase of NO was suppressed by adding 1400w which is a highly selective iNOS inhibitor. This suggested that the increase of NO was attributed to the improvement of iNOS activity and mRNA expression. Peluso et al. (1994) found that chitosan could also stimulate rat macrophages 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 RAW 264.7 cells. Our results were similar to those of previous studies. In addition, we also found that chitosan could markedly inhibit the NO production, iNOS activity, and mRNA expression of PBMCs induced by LPS. Yang et al. (2010) indicated that chitosan markedly inhibited the LPS-induced NO production of RAW 264.7 cells. It was reported that chitosan inhibits the production of NO in LPS-activated RAW 264.7 cells, but stimulates the release of NO in resting or unstimulated peritoneal macrophages (Hwang et al. 2000; Chou et al. 2003). Therefore, it is concluded that the effects of chitosan on PBMCs seem to depend on the activation status of cells: chitosan inhibits the production of NO in LPS-induced PBMCs but stimulates the release of NO in unstimulated PBMCs, which exerted bidirectional regulatory effects on the production of NO by modulated iNOS activity and mRNA expression in piglet PBMCs.

CONCLUSION

In a word, our results indicated that the NO concentrations, iNOS activity, and mRNA expression in pigs were increased by feeding chitosan in a dose-dependent manner. In addition, chitosan improved the NO production in unstimulated PBMCs but inhibited its production in LPS-induced PBMCs, which exerted bidirectional regulatory effects on the production of NO via modulated iNOS activity and mRNA expression in piglet PBMCs. Our results open the possibility that in the near future, this or other polysaccharides could be used as an additive to boost piglet immune functions. However, more research is required to further explore the mechanism by which different levels of chitosan affect immune functions in weaned piglets.

Acknowledgement. The authors gratefully acknowledge the support of the National Natural Science Foundation of China. The animal trial in this study was supported by Jin Feng Gu Agricultural Company in Erdos, China.

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Received: 2014–01–19
Accepted after corrections: 2015–01–16

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