Developmental Gene Expression of Antimicrobial Peptide PR-39 and Effect of Zinc Oxide on Gene Regulation of PR-39 in Piglets*

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ABSTRACT : Two experiments were conducted to evaluate developmental gene expression of antimicrobial peptide PR-39 and effect of zinc oxide on gene regulation of PR-39 in piglets using semi-quantitative RT-PCR analysis. In experiment 1, fifteen female Tai-Hu pigs (a local breed in China) in five groups, each of three pigs at 1, 14, 28, 42 and 56 days of age were used to determine effect of age and weaning on mRNA expression of PR-39. In experiment 2, nine groups of pigs (total seventy-two female 36 days-age weanling Tai-Hu piglets) were assigned to three treatments (ZnO_0, ZnO_{100} and $ZnO_{3,000}$). The feeding experimental period lasted 15 days. After feeding experiment, nine pigs with three animals in each treatment were chosen to determine the effect of ZnO on PR-39 mRNA expression of pigs. The results showed that PR-39 mRNA levels increased steadily in postnatal day 1-28 (preweaning), and weaning significantly decreased PR-39 mRNA expression of piglets ($p<0.05$). $ZnO_{3,000}$ (3,000 mg zinc/kg diet) significantly increased PR-39 mRNA expression ($p<0.05$) when piglets were feed $ZnO_{3,000}$ diet for 15 days. ZnO_{100} (100 mg zinc/kg diet) also increased PR-39 gene expression, but the result was not statistically significant ($p>0.05$). The result was in accordance with the effect of $ZnO_{3,000}$ and ZnO_{100} on weight gain of piglets and prevention of diarrhea. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 12 : 1635-1640)***

Key Words : PR-39, Piglet, Gene Expression, Zinc Oxide, RT-PCR, Antimicrobial Peptide

INTRODUCTION

Antimicrobial peptides are an important first line of defense against microbial invasion and play a prominent role in host defense mechanisms of innate immunity (Boman, 1995; Nicholas and Mor, 1995; Boman, 1998; Lehrer and Ganz, 1999). Among these natural antibiotics are a family of antimicrobial peptides called cathelicidins (Zanetti et al., 1995). Cathelicidins are the largest family of antimicrobial peptides in pigs and include PR-39, a proline-arginine-rich 39-amino-acid residue antimicrobial peptide (Storici and Zanetti, 1993; Gudmundsson et al., 1995; Zhang et al., 2000); protegrins 1 to 5 (Zhao et al., 1994) and prophenins 1 and 2 (Harwig, 1995; Zhao et al., 1995).

In general, PR-39 is synthesized by bone marrow progenitor cells (Zanetti et al., 1995), stored as proforms in neutrophil granules (Zanetti et al., 1990), and processed to mature peptides by enzyme cleavage (Panyutich et al., 1997). Although Wu et al. (1999) investigated the gene expression of porcine PR-39 during the first month of age, the difference of PR-39 mRNA expression at each of the ages was not reported, and especially the difference of PR-39 mRNA expression preweaning and postweaning was not further studied. In addition, although *in vitro* induction of antimicrobial peptides has been reported previously for cathelicidin (Wu et al., 2000), information on the *in vivo* modulation of cathelicidin gene expression is sparse. Up to now, the effect of necessary micro-mineral elements on cathelicidin gene expression has not been reported. The presence of potential binding sites of zinc finger peptides in the promoter region of PR-39 suggests that it is likely that PR-39 is induced by zinc (Zhao et al., 1995).

In the current study, two experiments were conducted to determine the effect of age and weaning on PR-39 gene expression, and whether feeding zinc (from zinc oxide) can affect PR-39 expression *in vivo*.

MATERIAL AND METHODS

Experiment 1

Animals : Total of fifteen female Tai-Hu pigs (a local breed in China, mean birth weight=1.31±0.05 kg) in five groups, each of three pigs at 1, 14, 28, 42 and 56 days of age were euthanized under anesthesia for sampling. These healthy pigs from three same breed litters were fed in a conventional environment before euthanasia. The piglets had been weaned at 36 days after birth. All the animal experiments were done according to the guidelines for animal experiments at the National Institute of Animal Health.

RNA extraction : Femurs of all pigs were dissected, and bone marrow cells were aspirated. Total RNA was extracted from bone marrow cells by using TRIzol (Gibco, BRL) according to the manufacture's manual. Extracted RNA was resuspended in 20 μl ultra-pure water. The purity and concentration were checked using a spectrophotometer at 260 and 280 nm.

RT-PCR : RT-PCR was performed in a thermocycler

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Table 1. Primer sets for RT-PCR of porcine PR-39 and β-actin

Gene	Oligonucleotide sequence	Size of PCR product
PR-39	5'-cgctggtcactgtggcttct-3'(S)	285
	5'-ctgcttcactcgcccattct-3'(AS)	
β-actin	5'-cgggacctgaccgactacct-3'(S)	411
	5'-ggccgtgatctccttctgc-3'(AS)	

(Gene Amp PCR system 9600). Two micrograms of RNA isolated from each sample was reverse transcribed using oligo $(dT)_{15}$ primers (Promega. Madison. USA) and an RNA PCR kit (AMV) (Promega. Madison. USA). essentially according to the manufacturer's protocol. The cDNA equivalent to 160 ng total RNA was subjected to PCR using Taq DNA Polymerase (Promega. Madison. USA) in a 50 volume containing 2.5 mM Mg^{2-}. 0.4 μM each sense and antisense primer. The PCR primer sets used are shown in Table 1. Primer sequences for PR-39 and β-actin housekeeping gene were designed by using the Primer Program of the Winsconsin Sequence Analysis Package (Genetics Computer Group. inc.) based on known sequences deposited in Genebank. The optimum PCR reaction cycles and concentration of Mg^{2+} to give a linear amplification of each transcript were determined by a preliminary experiment (Data not shown). PCR for PR-39 andβ-actin was done under the following thermal cycles: PR-39 at 94°C for 45 s. 57.5°C for 45 s and 72°C for 1 min for 29 cycles: β-actin at 94°C for 45 s. 57.5°C 45 s and 72°C 1 min for 29 cycles.

Experiment 2

Animals and diet : Seventy-two female 36 days-age weanling Tai-Hu pigs (a local breed in China, mean birth weight=1.35±0.04 kg) with an average initial body weight of 9.83±0.25 kg were randomly allocated to nine groups of eight animals each. The animals were fed a diet based on corn grain, wheat and soybean meal supplemented with lysine, methionine. vitamins and minerals to fulfil the pigs' requirement for all nutrients except zinc. Representative samples of the basic diet were analysed chemically. and the composition and analysed nutrient contents are shown in Table 2.

Nine group pigs were assigned to three treatments (each consisting of three group replicates): inclusion of 0. 100. 3,000 mg zinc (as zinc oxide) to the basic diet that contained 42 mg zinc per kg DM (analyzed). These three treatments were designated ZnO_0. ZnO_{100}, and $ZnO_{3,000}$. The basic diet was made as one batch and was not pelleted. Initially. the calculated amounts of zinc oxide were carefully premixed with small portions of maize starch. and these premixes were then finally mixed into the feed previously supplied with the recommended levels of all other nutrients. Representative samples of the zinc supplemented diets were analyzed for zinc content (Table 2).

Feed and water were offered *ad libitum* and the feeding experimental period lasted 15 days. After feeding experiment. the body weight of the animals and total feed intakes of each group pigs were recorded. Nine pigs with three animals each treatment (one animal each group) were chosen to be euthanized under anesthesia for sampling. Femurs of all pigs were dissected. and bone marrow cells were aspirated.

RNA extraction : The method of RNA extraction as

Table 2. The composition and analysed nutrient content of the diet

Composition	ZnO_0	ZnO_{100}	$ZnO_{3,000}$
Corn grain (%)	54	53.99	53.7
Wheat (%)	6	6	6
Soybean meal (%)	31.3	31.3	31.3
Whey (%)	3	3	3
Soybean oil (%)	2	2	2
Dicalcium phosphate (%)	0.9	0.9	0.9
Calcium carbonate (%)	1.2	1.2	1.2
Sodium chloride (%)	0.3	0.3	0.3
Lysine, 76% (%)	0.2	0.2	0.2
Methionine, 98% (%)	0.1	0.1	0.1
Mineral and vitamin premix[a] (%)	1	1	1
Supplementation of Zn as ZnO (%)	0 (0 mg/kg)	0.01 (100 mg/kg)	0.3 (3,000 mg/kg)
Analysed content (per kg dry matter) :			
Crude protein (N·6.25) (g)	201	201	200
Crude fat (g)	41	42	40
Crude fibre (g)	20	21	19.8
Calcium (g)	7.6	7.8	7.7
Available phosphorus (g)	3.2	3.0	3.1
Zinc (mg)	42	150	3,065

[a] Mineral/vitamin supplied per kilogram: Provided the following amounts of trace minerals per kilogram of complete diet: Fe as $FeSO_4$. 110 mg: Cu as $CuSO_4$, 22 mg: Mn as $MnSO_4$, 55 mg: I as KI. 0.28 mg: Se as $NaSeO_3$, 0.3 mg. Vit. A 8,000 IU; Vit. D_1. 500 IU; Vit. E 30 mg: Vit. B_1 4.4 mg; Vit. B_2 4 mg: Vit B_6 2 mg; Vit. B_{12} 0.22 mg: Niacin 22 mg; d-Pantothenic acid 5 mg; Biotin 0.15 mg: Folic acid 0.35 mg.

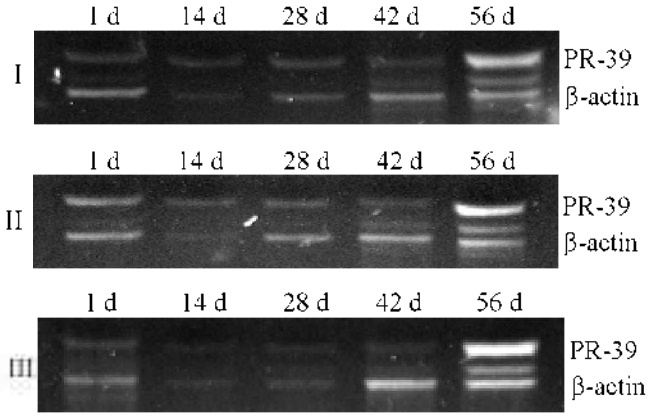

Figure 1. Electrophoresis results of RT-PCR for PR-39 and β-actin in the bone marrow of three piglets of the 1-day-old, 14-day-old, 28-day-old, 42-day-old and 56-day-old. I, II and III: The results from the first piglet, the second piglet and the third piglet in each age groups respectively.

described in Experiment 1.

RT-PCR : The method of RT-PCR as described in Experiment 1.

Data analysis

Electrophoresis band intensities of the PCR products were quantified using NIH Image Version 1.62 software. Mean PR-39 mRNA expression levels normalized against β-actin levels from each age group were presented in arbitrary units. Each value was analyzed for statistical difference according to the Bonferroni/Dunn method (Duncan, 1955). The daily weight gain and feed/gain of three treatment pigs for statistical calculations were also performed by use of the Bonferroni/Dunn method.

RESULTS AND DISCUSSION

In the Experiment 1, we compared antimicrobial peptide PR-39 mRNA expression of piglets in different ages by semi-quantitative PCR. RT-PCR allows the relative quantification of PR-39 mRNA levels of bone marrow in different ages, although not the absolute quantification. The electrophoresis results of three pigs of each age group are shown in Figure 1.

Developmental changes in PR-39 mRNA levels were evaluated in postnatal day 1-56 female piglet marrow using semi-quantitative RT-PCR analysis. The resulting 417 bp amplicon has been previously sequenced from pig marrow (Wang et al., 2002) and was 100% homologous to the known sequences of PR-39 deposited in Genebank.

As shown in Figure 1, PR-39 mRNA was detected in bone marrow at all stages of neonatal development (postnatal day 1-56), and the difference in PR-39 expression were age-dependent. PR-39 mRNA levels increased steadily in postnatal day 1-28 (preweaning), however PR-39 mRNA

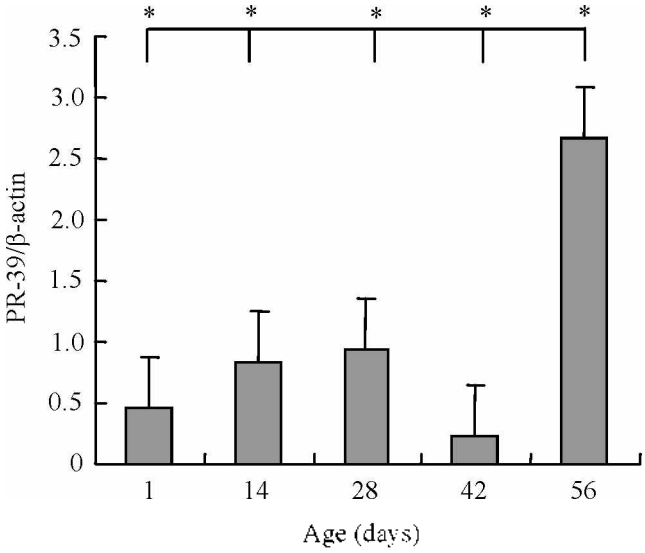

Figure 2. mRNA expression of PR-39 in bone marrow of 1-day-old, 14-day-old, 28-day-old, 42-day-old and 56-day-old pigs. Bar show the mean expression of mRNA of three pigs as the ratio of the band intensity of each PCR product to the corresponding β-actin PCR product. * $p \leq 0.05$.

levels of postweaning pig (postnatal day 42) were significantly lower than preweaning pigs (postnatal day 28) when piglets were weaned at postnatal day 36. In the later postweaning period (postnatal day 56), stronger PR-39 mRNA expression was observed (Figure 2). The result from Wu et al. (1999) also indicated that bone marrow cells showed abundant expression of PR-39 in pigs at the first month of age, but they did not investigate the effect of weaning on PR-39 mRNA expression of piglets. In the current study, the result implied that PR-39 expression was not only age-dependent but also that weaning significantly decreased the gene expression of antimicrobial peptide PR-39 in piglets.

Weaning can often be a time associated with a lag in performance (referred to as "postweaning lag") that includes depressed gain, feed intake and diarrhea with increased disease and mortality. Weaning is probably the most stressful time in a pig's life because of the many changes it must undergo. One of these changes is immunological change. The newborn pig acquires passive immunity by absorbing the antibodies present in colostrum. The levels of these maternally-derived antibodies are highest on day one post-farrowing and then decline to very low levels by the time the pig reaches about three weeks of age. The pig's own immune system begins developing at approximately three weeks of age, but is not able to mount an effective active immune response until the pig is up to five weeks of age. However the piglets are usually weaned at four- to five-weeks-old, and this makes the newly weaned pig very susceptible to disease and pathogenic stressors. Therefore, components of the gut mucosal barrier or non-

Table 3. Effect of zinc oxide on growth performance of newly weaned pigs

	ZnO_0 (No adding Zn)	ZnO_{100} (Adding 100 mg Zn/kg)	$ZnO_{3,000}$ (Adding 3,000 mg Zn/kg)	SEM
Initial weight (kg)	10.04	9.73	9.71	0.12
Final weight (kg)	14.79	15.06	16.51	0.29
ADG (g)[d]	317[b]	355[b]	454[a]	12.29
ADFI (g)[d]	592[b]	596[b]	799[a]	14.17
Feed/gain	1.88	1.68	1.76	0.07

[d] Means within in the same row without a common superscript differ ($p<0.01$).

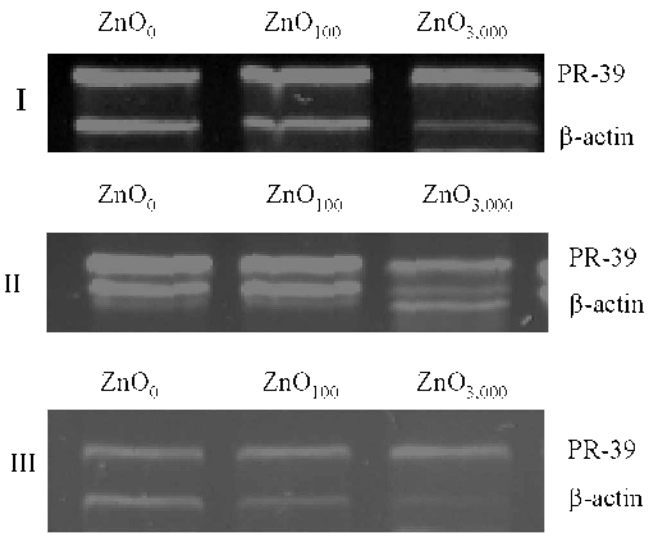

Figure 3. Electrophoresis results of RT-PCR for PR-39 and β-actin in the bone marrow of three piglets of the ZnO_0, ZnO_{100} and $ZnO_{3,000}$. I, II and III: The results from the first piglet, the second piglet and the third piglet in each age groups respectively.

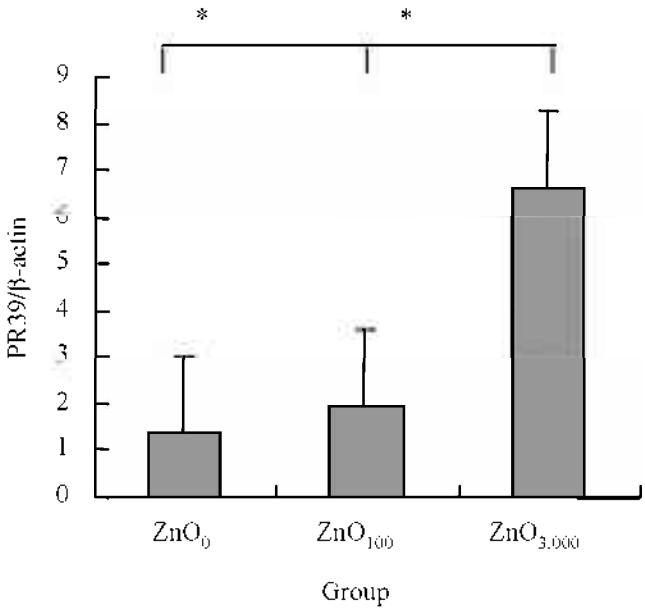

Figure 4. mRNA expression of PR-39 in bone marrow of ZnO_0, ZnO_{100}, and $ZnO_{3,000}$ group pigs. Bar show the mean expression of mRNA of three pigs as the ratio of the band intensity of each PCR product to the corresponding β-actin PCR product. * $p\leq0.01$.

specific immune factors (e.g. antimicrobial peptides) is more important to the weanling pig's growth and immunity in weaning phase (Bosi et al., 2003).

The results from the current experiment implied that weaning resulted in decreased expression of non-specific immune factor (e.g. antimicrobial peptide PR-39) not only lack of colostral or milk antibodies of piglets, and further caused postweaning diarrhea and disease. After piglets were weaned, a nutrient weaning diet will be supplemented to weanling pigs. So stronger expression of PR-39 in the later postweaning (postnatal day 56) may reflect the positive effect of nutrition on its expression or the developed maturation process of antimicrobial peptide PR-39 expression itself.

In experiment 2, a feeding trial was conducted to study the effect of zinc oxide on growth performance in weaned pigs. Results from the current experiment showed that $ZnO_{3,000}$ increased ADG (Average Daily Gain) and ADFI (Average Daily Feed Intake) by 27.9% ($p<0.01$) and 34.1% ($p<0.01$) respectively compared with ZnO_{100}, and increased ADG and ADFI by 43.2% ($p<0.01$) and 35.0% ($p<0.01$) respectively compared with ZnO_0 (see Table 3). In addition, $ZnO_{3,000}$ can effectively decrease postweaning diarrhea (result was not shown in Table 3).

Zinc (Zn) is an essential trace mineral for swine. The requirement for Zn has been suggested to be in the range of 50 to 100 mg/kg for pigs at various stages of growth (NRC, 1998). The bioavailability of zinc oxide (ZnO) as a source of Zn is lower than other Zn sources such as zinc sulfate ($ZnSO_4$), zinc carbonate ($ZnCO_3$), and Zn-methionine in weanling pigs. Some previous studies have shown that supplementation of 50 mg/kg or 150 mg/kg of Zn to corn-soybean meal based diets did not improve the growth performance and immunity of growing pigs (Roberts et al., 2002), but adding pharmacological levels (3,000 mg/kg) of Zn as ZnO to corn-soybean meal based diets significantly improved starter pig performance and was more effective in controlling diarrhea for weanling pigs (Hill et al., 2000; Case and Carlson, 2002). Results from the current experiment were in agreement with these previous studies. These results implied that $ZnO_{3,000}$ significantly improved growth performance by effectively controlling E.coli scours and enhancing immunity for weanling pigs (Carlson et al., 1999).

We further investigated the effect of zinc on gene expression of antimicrobial peptide PR-39 *in vivo* and semi-quantitative RT-PCR was used to evaluate PR-39 mRNA expression. The current result show that $ZnO_{3,000}$ (3,000 mg zinc/kg diet) significantly increased PR-39 mRNA expression when piglets were feed $ZnO_{3,000}$ diet for 15 days. ZnO_{100} (100 mg zinc/kg diet) also increase PR-39 gene expression, but was not statistically significant. (see Figures 3 and 4)

The result from Wu et al. (1999) suggested the existence of regulatory elements controlling PR-39 expression in newborns and raised the possibility of altering these control elements to modulate PR-39 expression. In 2,000. Wu et al. (1999) reported that Lipopolysaccharide (LPS). Interleukin-6 (IL-6) and Retinoic Acid (RA) upregulate PR-39 gene expression.

The potential importance of zinc to the gene can be appreciated from the fact that about 25% of the zinc content of rat liver is found in the cell nucle (Cousins. 1998). Zinc is involved in the processes of genetic stability and gene expression in a variety of ways including the structure of chromatin. the replication of DNA and transcription of RNA (Falchuk. 1998). Recently. increasing emphasis has been placed on the role played by zinc in zinc-finger proteins. which are mainly nuclear transcription factors which. together with other families of transcription factors (i.e. homeobox, leucine zipper and helix loop), control cell proliferation, differentiation and apoptosis through regulation of gene expression (Klug. 1999). Zhao et al. (1995) reported that the 5' upstream regions of the PR-39 gene contained clusters of potential transcriptional regulatory elements. including NK-κB and NF-IL-6 and the binding site with zinc finger protein. These reports implied the possibility of zinc modulating PR-39 expression. Results from in the current experiment also indicate that zinc can upregulate PR-39 mRNA expression. The effect of $ZnO_{3,000}$ on PR-39 gene expression is more significant than ZnO_{100}. It is in accordance with the effect of $ZnO_{3,000}$ on weight gain of piglets (Table 3) and preventing diarrhea (Hill et al.. 2001). Although the result from the current experiment showed that $ZnO_{3,000}$ also significantly increased average daily feed intake (ADFI) of weaning pigs. it was not clear that whether $ZnO_{3,000}$ markedly upregulated gene expression of PR-39 is positively related to significantly increasing ADFI of weaning pigs. Antimicrobial peptides PR-39. as important non-specific immune factors. play a prominent role in host defense mechanisms of innate immunity. Whether $ZnO_{3,000}$ can significantly prevent diarrhea and promote growth of piglets by markedly enhancing PR-39 gene expression should be further investigated.

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