Dietary Zinc Effects on Growth Performance and Immune Response of Endotoxemic Growing Pigs**

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ABSTRACT : A 2×3 factorial arrangement of treatments was used in a completely randomized design to determine the effects of dietary Zn on performance and immune response of acutely endotoxemic growing pigs (n=96, mean BW=24.9 kg). Factors included 1) intramuscular injection of 10 μg/kg BW of *Escherichia coli* lipopolysaccharide (LPS) or control and 2) supplemental Zn at 10, 50, or 150 ppm. Diets were fed beginning after weaning (initial body weight=7.6 kg) in the nursery and continued for 16 d into the grower phase. The basal corn-soybean meal grower diet contained 1% lysine and 34.3 ppm Zn. Pigs were acclimated for 12 d in the grower-finishing facility before LPS treatment on d 13. Gain, feed intake, and feed efficiency were unaffected by dietary Zn. Feed intake decreased ($p<0.10$) and gain/feed was greater ($p<0.10$) from d 13 to d 16 for pigs injected with LPS. Serum Zn and alkaline phosphatase activity increased ($p<0.05$) with increasing Zn levels. The febrile response to LPS peaked at 6 h post exposure and pigs were afebrile within 12 h. Rectal temperature was greater ($p<0.05$) in pigs receiving 50 and 150 ppm Zn than in pigs supplemented with 10 ppm Zn. In vivo cellular immune response, measured on d 13 by skin thickness response to phytohemagglutinin (PHA), was greater after 6 h ($p<0.05$) in pigs fed 10 ppm Zn and exposed to LPS compared to all other treatments, but was not affected at 12, 24 or 48 h. Zinc did not affect mitogen induced lymphocyte proliferation. Zinc supplemented at 50 or 150 ppm resulted in an enhanced febrile response in pigs subjected to iatrogenic endotoxemia, but did not affect pig performance or immune response measurements. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 10 : 1496-1501)***

Key Words : Pigs, Zinc, Immune Response

INTRODUCTION

Zinc is a micromineral that is crucial for growth and immune system function (Chesters, 1997) and has been successfully used to restore impaired immune function when supplemented during a Zn deficiency (Vallee and Falchuk, 1993). However, when supplemented in excess, Zn has been reported to reduce lymphocyte stimulation response to phytohemagglutinin (PHA), impair chemotaxis and phagocytosis of bacteria by polymorphonuclear leukocytes in humans (Chandra, 1984), increase plasma levels of tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) in pigs (Klosterhalfen et al., 1996) and exaggerate the acute phase response in humans (Braunschweig et al., 1997).

Zinc concentrations in serum and plasma have been demonstrated to decrease following experimental infection (Tufft et al., 1988; Hill, 1989) or intravenous injection with endotoxin (Butler and Curtis, 1973) in poultry. Administration of endotoxin reduced plasma Zn concentration and flux of Zn through plasma in pigs fed diets adequate in Zn (Chesters and Will, 1981). The potential role that Zn may play during disease or immunologic challenge in pigs has not been studied, and the requirement for Zn may be different under these circumstances than the requirement for growth in healthy pigs as suggested by NRC (1998). Therefore, the objective of this experiment was to characterize the effect of dietary Zn concentration on performance and immune response of acutely endotoxemic growing pigs.

EXPERIMENTAL PROCEDURES

General procedures

The experimental protocols used in this study were approved by the North Carolina State University Institutional Animal Care and Use Committee. Ninety-six crossbred pigs were weaned at 21 d of age (average initial BW was 7.6 kg), stratified by weight, and then randomly assigned to one of six treatment groups. Pigs were housed eight pigs per pen in the nursery for six weeks. After six weeks, pigs (BW=24.9 kg) were moved to a grower-finisher facility and, within a treatment group, pigs were housed in groups of similar weight at four pigs per pen, using 24 pens. A 2×3 factorial arrangement of treatments was used in a completely randomized design to determine the effects of dietary Zn and *Escherichia coli* lipopolysaccharide (LPS) administration on performance, serum Zn, alkaline

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phosphatase activity (ALP), and immune response. Factors included: 1) intramuscular injection of 10 μg/kg BW of LPS (serotype 055:B5, Sigma Chemical, St. Louis, MO) or sham injection with physiological saline and 2) supplemental Zn supplied as $ZnSO_4$ at 10, 50, or 150 ppm. Diets (described below) were fed beginning in the nursery and were continued for 16 days into the grower period. Pigs were acclimated for 12 d (d 0 to 12) in the grower-finisher facility before LPS injection on d 13. Pigs were given ad libitum access to feed and water. Body weight and feed intake were determined prior to injection with LPS or saline and at 3 d after injection. In addition, blood samples for ALP and serum Zn determination were obtained by venipuncture. General animal health was noted daily by monitoring attitude, clinical appearance, and behavior. Rectal temperatures were obtained from one randomly selected pig per pen at time of LPS injection and again in the same pigs at 6, 12, 24, and 48 h after injection.

Experimental diets

Experimental diets (Table 1) were formulated to meet or exceed NRC (1998) recommendations, with the exception of dietary Zn. Zinc was supplemented as $ZnSO_4$ at 10, 50, or 150 ppm to a corn-soybean meal basal diet containing 34.3 ppm Zn. These levels were chosen to reflect Zn levels below and at the NRC minimum recommended level of 60 ppm, and to further evaluate the industry standard practice of grower-finisher supplementation of Zn above NRC requirements. Analyzed Zn concentrations in experimental diets were 34.3, 62.3, and 113.5 ppm for the prestarter diets, 38.2, 59.4, and 154.4 ppm for the starter diets and 42.8, 70.5, and 131.9 ppm for the grower diets for the 10, 50, and 150 ppm supplemental Zn treatments, respectively. Dietary Zn and serum Zn concentrations were measured by atomic absorption spectrophotometry (Shimadzu, Model AA-6701F, Kyoto, Japan). Serum ALP activity was measured using Sigma Diagnostics Alkaline Phosphatase reagent (ALP 50, Sigma Chemical) which measures ALP activity by a kinetic method similar to the procedure described by Bowers and McComb (1966).

Table 1. Composition of the basal diets[a]

	Pre-starter	Starter	Grower
Ingredient, %			
Corn	42.62	59.17	68.05
Soybean meal (48% CP)	22.00	32.89	24.47
Blood plasma	2.50	-	-
Fish meal (menhaden)	6.91	-	-
Whey	20.00	-	-
L-lysine-HCl	0.05	0.07	0.11
DL-methionine	0.11	0.04	0.01
Dicalcium phosphate	0.44	1.66	1.30
Limestone	0.52	0.92	0.81
Salt	0.10	0.50	0.50
Vitamin-mineral premix[b]	0.25	0.25	0.25
Antibiotic[c]	0.50	0.50	0.50
Poultry fat	4.00	4.00	4.00
Calculated composition, %			
Crude protein	22.81	20.63	17.36
Lysine	1.50	1.20	1.00
Ca	0.90	0.85	0.70
P	0.75	0.70	0.60

[a] As-fed basis.

[b] Provided the following amounts of vitamins and trace minerals per kilogram of complete diet: vitamin A as retinyl acetate, 5,510 IU; cholecalciferol, 1,102 IU; DL-tocopherol, 22 IU; vitamin B_{12}, 0.022 mg; riboflavin, 4.4 mg; niacin, 22 mg; d-pantothenic acid as dl-calcium pantothenate, 17.6 mg; menadione dimethylpyrimidinol bisulphite, 4.4 mg; choline as choline chloride, 220 mg; folic acid, 0.33 mg; thiamine as thiamin mononitrate, 0.55 mg; pyridoxine as pyridoxine-HCL, 1.1 mg; d-biotin, 0.04 mg; I as EDDI, 0.28 mg; Se as $NaSeO_3$, 0.3 mg; Cu as $CuSO_4$, 25 ppm; Fe as $FeSO_4$, 180 ppm; Mn and $MnSO_4$, 60 ppm.

[c] Supplied as 55 mg carbadox/kg of complete diet.

Immune response measurements

Cellular immune response was measured *in vivo* on d 13 using a PHA (Sigma Chemical) skin test (Kornegay et al., 1989). One randomly selected pig per pen was injected subcutaneously in the skinfold of the right flank with 0.1 mL of PHA (150 μg/mL). Skinfold thickness was determined using calipers at 0, 6, 12, 24 and 48 h after injection of PHA.

In vitro cellular immune response was measured on d 14 in one pig per pen, randomly selected from the three pigs per pen not receiving the PHA skin test, using a lymphocyte blastogenesis assay (Blecha et al., 1983). Approximately 15 mL of blood was collected into heparinized tubes by venipuncture for isolation of mononuclear cells. Blood mononuclear cells were isolated by gradient centrifugation and plated in 96 well plates (Corning, Corning, NY) at a concentration of 2×10^6 cells/mL. The cells were treated with the mitogens, PHA and pokeweed mitogen (PWM, Sigma Chemical), at a concentration of 10 μg/mL each. These mitogen concentrations were shown to provide near maximum stimulation of blood mononuclear cells (Morrow-Tesch et al., 1994; van Heugten et al., 1994). Cells were incubated at 37°C in 5% CO_2 atmosphere for 48 h. Cultures were then pulsed with $\{^3H\}$thymidine (6.7 Ci/mmol, ICN Radiochemicals, Irvine, CA), incubated for an additional 18 h, and collected on glass fiber filter strips using an automated cell harvester (PHD cell harvester, Cambridge Technology, Watertown, MA). Uptake of $\{^3H\}$thymidine served as the measure of cell proliferation.

Statistical analyses

Data were analyzed as a completely randomized design with a 2×3 factorial arrangement of treatments using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included LPS, Zn, and the LPS×Zn interaction.

Differences in temperature were analyzed using repeated measures. Pen means were used to analyze pig performance, whereas individual pig data served as the experimental unit in the immune response data. Differences between treatment means were determined using a *t*-test following a significant *F*-test.

RESULTS AND DISCUSSION

Vomiting, diarrhea, anorexia, lethargy, dyspnea, and depression were noted within 30 min of LPS injection. The anorexia and fever seen in the LPS treated pigs are consistent with previous research (Lee et al., 2000) and demonstrate that we were able to successfully challenge the immune system. The febrile response (Figure 1) to LPS peaked at 6 h post exposure and pigs were afebrile within 12 h. At 6 h, rectal temperature was greater ($p<0.05$) in pigs receiving 50 and 150 ppm Zn than in pigs fed 10 ppm added Zn. Our results concur with Braunschweig et al. (1997) who reported that dietary Zn administration in septic patients exaggerated the acute phase response as evidenced by higher febrile responses. The enhanced febrile response seen in 50 and 150 ppm supplemental Zn groups may have occurred because Zn, in addition to LPS, reportedly induces cytokine release, predominantly IL-1β, IL-6, and TNF-α (Driessen et al., 1994; Johnson, 1997). These cytokines are known for their pyrogenic effects. Zinc-induced cytokine production is thought to be caused by a direct interaction of Zn with monocytes (Wellinghausen et al., 1997). Furthermore, Driessen et al. (1995) reported that Zn enhanced LPS activity so strongly that unstimulatory doses of LPS combined with unstimulatory doses of Zn resulted in activation of monocytes. Therefore, Zn probably augments proliferation as well as cytokine secretion in monocytes and could influence the immuno-stimulative effects of LPS.

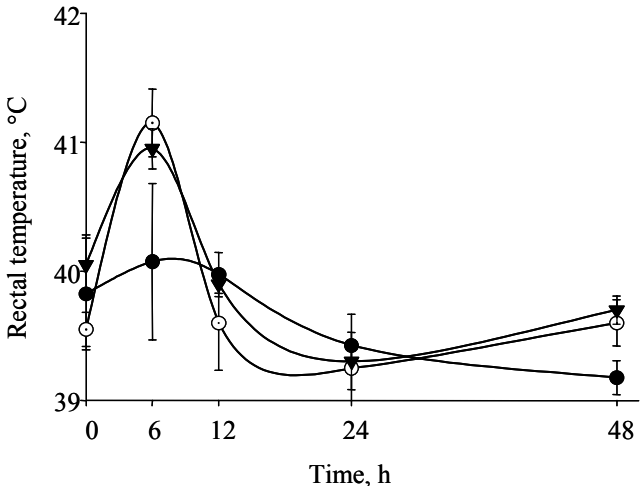

Figure 1. Rectal temperature changes in pigs injected with lipopolysaccharide (●, 10 ppm supplemental zinc (n=12); ○, 50 ppm supplemental zinc (n=12); ▼, 150 ppm supplemental zinc (n=12). Maximum rectal temperature was greater ($p<0.05$) in pigs receiving 50 and 150 ppm zinc than in pigs with 10 ppm added zinc. Rectal temperatures were 40.1, 39.8, 39.7 and 39.4°C at 0, 6, 12, and 24 h after saline injection in control pigs, respectively.

Feed intake decreased ($p<0.10$) and gain/feed was greater ($p<0.10$) from d 13 to d 16 for pigs injected with LPS, but pig performance was not affected by dietary Zn (Table 2). The lack of a depression in growth rate and the improvement in gain/feed following the LPS injection is surprising, particularly given the fact that visual observations of pigs and an increase in body temperature clearly indicated an acute inflammatory response. In addition, we have previously reported consistent reductions in growth, feed intake, and gain/feed in nursery pigs injected with LPS (van Heugten et al., 1994, 1996; van Heugten and Spears, 1997). The level of endotoxin injected in those studies was 200 µg/kg of BW compared to 10 µg/kg of BW in this study. Webel et al. (1997) reported that injection with 5 µg/kg BW of LPS was effective in increasing cytokine production. The level of 10 µg/kg BW of LPS in the present study was effective in eliciting a febrile response, but may have been too low to cause the reduction in performance reported previously. No interactions were observed ($p>0.10$) between LPS injection and dietary Zn supplementation for growth performance. Gain, feed intake, and feed efficiency were unaffected by dietary Zn ($p>0.10$) suggesting that there is no performance benefit to Zn supplementation above 10 ppm to a diet containing 34.3 ppm of Zn in control pigs or pigs injected with endotoxin.

Serum Zn and ALP increased ($p<0.05$) with increasing Zn levels (Table 3), which is in agreement with previous studies (Hoekstra et al., 1967; Liptrap et al., 1970). There was no effect of LPS injection on ALP or serum Zn concentration. This is in contrast to previous reports by Kincaid et al. (1976) and Chesters and Will (1981), who reported a reduction in serum Zn levels and ALP following bacterial endotoxin exposure. The hypozincaemia commonly observed during endotoxemia is transient and appears to follow changes in cytokine levels (Gaetke et al., 1997). In our study, a single dose of endotoxin increased body temperature for a period of less than 12 h and has further been reported to elevate TNF-α, IL-6, and cortisol for up to 8 h after injection (Webel et al., 1997). Therefore, serum Zn levels measured 72 h after pigs were injected with LPS may have returned to control levels by the time measurements were made.

In vivo cellular immune response was measured on d 13 by skin thickness response to PHA. The reponse to PHA has been shown to be of primarily mononuclear origin and is considered a valid measure of cellular immunity (Regnier

Table 2. Effect of lipopolysaccharide (LPS) challenge and dietary zinc on gain, feed intake, and efficiency of gain of growing pigs[a]

Item	Lipopolysaccharide[b]		SEM	Supplemental zinc, ppm			SEM
	-	+		10	50	150	
Gain, kg/d							
d 0-13	0.76	0.76	0.01	0.72	0.72	0.84	0.04
d 13-16	0.92	1.00	0.04	0.96	0.92	1.00	0.02
Feed Intake, kg/d							
d 0-13	1.60	1.72	0.06	1.56	1.64	1.76	0.06
d 13-16[c]	1.80	1.68	0.06	1.68	1.72	1.76	0.02
Gain:Feed							
d 0-13	0.48	0.44	0.02	0.46	0.44	0.48	0.01
d 13-16[c]	0.51	0.60	0.05	0.57	0.53	0.57	0.01

[a] Data are means of four pens of four pigs per pen.
[b] LPS was administered intramuscularly at 10 µg/kg BW on day 13 of trial.
[c] LPS effect ($p<0.10$).

Table 3. Effect of lipopolysaccharide (LPS) challenge and dietary zinc on alkaline phosphatase (ALP) and plasma zinc[a]

Item	Lipopolysaccharide		SEM	Supplemental zinc, ppm			SEM
	-	+		10	50	150	
ALP (U/L)							
d 0[b]	123.53	123.34	5.11	110.73[c]	130.54[d]	129.04[d]	6.33
d 13[b]	107.85	110.86	4.16	101.58[c]	112.73[cd]	113.76[d]	5.32
d 16[b]	101.64	98.84	3.93	92.08[c]	94.33[c]	114.31[d]	4.86
Serum zinc (ppm)							
d 0[b]	1.20	1.04	0.08	0.97[c]	1.04[c]	1.35[d]	0.10
d 13[b]	1.28	1.28	0.06	1.08[c]	1.25[c]	1.50[d]	0.08
d 16[b]	1.34	1.25	0.06	0.98[c]	1.08[c]	1.37[d]	0.08

[a] Each value represents the mean of forty-eight pigs (main effect of LPS) or sixteen pigs (main effect of zinc supplementation).
[b] Zinc effect ($p<0.05$).
[c,d] Means within a row lacking a common superscript differ ($p<0.10$).

Table 4. Effect of lipopolysaccharide (LPS) challenge and dietary zinc on skin thickness response to phytohemagglutinin[a]

Item	- LPS			+ LPS			SEM
Added Zn, ppm	10	50	150	10	50	150	
Skin thickness response, cm							
0 h	0.51	0.54	0.51	0.50	0.57	0.61	0.05
6 h[b,c]	0.64[d]	0.65[d]	0.64[d]	0.86[e]	0.60[d]	0.66[d]	0.06
12 h	1.16	1.30	1.36	1.23	1.04	1.45	0.13
24 h	1.14	1.20	1.50	1.30	1.05	1.22	0.18
48 h	0.80	0.88	1.00	0.80	0.65	0.85	0.12

[a] Each value represents the mean of four pigs.
[b] Zinc effect ($p<0.10$).
[c] LPS×Zinc interaction ($p<0.10$).
[d,e] Means within the same row differ ($p<0.05$).

and Kelley, 1981; Kelley et al., 1982; Blecha et al., 1983). The reactivity of stressed pigs to PHA has been reported to be delayed and less intense compared to that of control pigs that were not stressed (Ekkel et al., 1995). Using injection with LPS as a stressor did not affect PHA skin thickness response in the current experiment. However, skin thickness response was greater ($p<0.05$) in pigs fed 10 ppm Zn and exposed to LPS compared to all other treatments after 6 h, which may indicate a reduced initial inflammatory response to PHA in immune stressed pigs fed 50 or 150 ppm of supplemental Zn. No further differences in skin thickness response were noted ($p>0.10$), and therefore supplementation with Zn does not appear to improve *in vivo* cellular immune response in either control or immune challenged pigs.

In vitro cell mediated immune response (Table 5) was measured on d 14 using a lymphocyte blastogenesis assay. Lipopolysaccharide injection resulted in increased lymphocyte proliferation ($p<0.05$) in cells stimulated with PHA. This observation is comparable to previous studies in

Table 5. Effect of lipopolysaccharide (LPS) challenge and dietary zinc on lymphocyte blastogenic response to phytohemagglutinin (PHA) or pokeweed mitogen (PWM)[a]

Item	Lipopolysaccharide		SEM	Supplemental Zinc, ppm			SEM
	-	+		10	50	150	
Unstimulated	6.71	10.12	2.21	9.50	8.11	7.64	2.70
Stimulated							
PHA[b]	45.47	72.20	8.69	55.49	60.46	60.55	10.64
PWM	18.99	26.30	3.21	21.14	23.56	23.24	3.94

[a] Each value represents the mean of four pigs. Values are expressed as cpm×10^3.
[b] LPS effect ($p<0.05$).

which LPS was shown to stimulate lymphocytes (Whitworth et al., 1989; Grabarek et al., 1990; van Heugten et al., 1994). Zinc did not affect lymphocyte proliferation ($p>0.10$). In contrast, Driessen et al. (1994) reported a stimulative influence of Zn on T-cells, which was suggested to represent an indirect effect mediated through the release of IL-1 by monocytes and cell to cell contact. Conversely, high Zn concentrations can inhibit T cell proliferation by blocking the IL-1 type I receptor associated kinase (Wellinghausen et al., 1999). Similarly, Chandra (1984) demonstrated a reduction in lymphocyte stimulation response to PHA as well as reduced chemotaxis and phagocytosis of bacteria by polymorphonuclear leukocytes with excessive Zn supplementation. Based on the present study, Zn supplementation at 10 to 150 ppm to a diet containing 30 ppm of Zn did not affect lymphocyte blastogenesis.

IMPLICATIONS

Our results indicate that Zn supplemented at 50 and 150 ppm to a corn-soybean meal based diet containing 30 ppm of Zn enhanced the febrile response in pigs subjected to iatrogenic endotoxemia. Gain, feed intake, feed efficiency and cellular immune response measurements were not affected by Zn supplementation; therefore supplementation above NRC recommendations for Zn is not beneficial. The amount of lipopolysaccharide injected in this study was effective in inducing an inflammatory response and slightly reduced feed intake, but did not appear to be effective in decreasing pig growth performance measurements.

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