Effects of Dietary Zinc on Performance and Immune Response of Growing Pigs Inoculated with Porcine Reproductive and Respiratory Syndrome Virus and *Mycoplasma hyopneumoniae**

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ABSTRACT : The objective of this study was to determine the effects of dietary Zn level on performance, serum Zn concentrations, alkaline phosphatase activity (ALP), and immune response of pigs inoculated with Porcine Reproductive and Respiratory Syndrome virus (PRRSv) and *Mycoplasma hyopneumoniae*. A 2×4 factorial arrangement of treatments was used in a randomized design. Factors included; 1) PRRSv and *M. hyopneumoniae* inoculation (n=36 pigs) or sham inoculation (n=36 pigs) with media when pigs entered the grower facility (d 0) at 9 weeks of age and 2) 10, 50, 150 ppm supplemental Zn sulfate ($ZnSO_4$) from weaning until the completion of the study, or 2,000 ppm supplemental $ZnSO_4$ for two weeks in the nursery and then supplementation with 150 ppm $ZnSO_4$ for the remainder of the trial. The basal diet contained 34 ppm Zn. Pigs were weighed on d 0, 10, 17, 24 and 31 and blood samples were collected on d 0, 7, 14, 21 and 28. Pigs inoculated with PRRSv were serologically positive at d 28 and control pigs remained negative to PRRSv. In contrast, the *M hyopneumoniae* inoculation was inconsistent with 33.3% and 52.8% of pigs serologically positive at d 28 in the control and infected groups, respectively. A febrile response was observed for approximately one week after inoculation with PRRSv. Feed intake ($p<0.01$) and gain ($p<0.1$) were less in PRRSv infected pigs than control pigs for the 31 d study. However, performance did not differ among pigs in the four levels of $ZnSO_4$. Assessments of immune responses failed to provide unequivocal influence of either PRRSv inoculation or $ZnSO_4$ level. These data suggest that PRRSv and *M. hyopneumoniae* act to produce some performance deficits and the influence of Zn supplementation of nursery age pigs does not have clear effect in grower pigs affected with disease. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1438-1446)***

Key Words : Pigs, PRRSv, Zinc, Growth, Immune Response

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSv) and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) commonly are involved in respiratory conditions of grow-finish pigs (Thacker et al., 1999). Both PRRSv and *M. hyopneumoniae* induce an immune response, albeit through different mechanisms (Messier et al., 1990; Benfield et al., 1999), and affected pigs have diminished weight gains and poor feed conversion (Kobisch and Friis, 1996).

Immunological challenge directs metabolism away from growth and skeletal muscle to support immune function (Spurlock, 1997). Most previous studies utilized endotoxin injections to stimulate the immune response (van Heugten et al., 1994b; Hevener et al., 1999). Unfortunately, the acute effects of endotoxin treatment rarely mimic the long-term, detrimental influence of infectious diseases in grow-finish pigs (Hevener et al., 1999).

* Supported in part by the College of Veterinary Medicine Research Program.
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Received December 19, 2003; Accepted June 4, 2004

Zinc is a crucial micromineral for growth and immune system function (Wellinghausen et al., 1997). Zinc supplementation successfully restored impaired immune function during malnutrition and acrodermatitis enteropathica (Vallee and Falchuk, 1993). However, when supplemented in excess, Zn reduced immune responses (Chandra, 1984) and exaggerated the acute phase response (Braunschweig et al., 1997) in humans and mice.

In pigs, several studies evaluated the effect of phase-feeding with pharmacological concentrations (2,000-3,000 ppm) of organic or inorganic Zn on performance of nursery pigs. In general, Zn supplementation improved pig growth (Carlson et al., 1999; Hill et al., 2000) and appeared to prevent postweaning diarrhea (Katouli et al., 1999). However, dietary excess increased excretion of Zn (Adeola et al., 1995), which may be viewed as a waste management concern.

The influence of Zn supplementation on growth and pig health has been determined for 14 to 28 d during the nursery phase of production in most studies. The potential benefits in subsequent phases of production typically were not measured despite the observation that Zn concentrations remained elevated in liver and renal tissue for an additional four to five weeks after cessation of Zn supplementation (Jensen-Waern et al., 1998). Therefore, the main objective of the present study was to determine the effects of dietary

Table 1. Composition of the basal diets[1]

Item	Pre-starter	Starter	Grower
Ingredient, %			
Corn	42.61	59.17	68.06
Soybean meal (48%CP)	22.00	32.89	24.47
Blood plasma, spray-dried	2.5	-	-
Fish, menhaden meal	6.91	-	-
Whey, dried	20.00	-	-
Lysine-HCl, 95%	0.05	0.07	0.11
Methionine	0.11	0.04	0.01
Dicalcium phosphate, 21%	0.44	1.66	1.30
Limestone	0.52	0.92	0.81
Salt	0.10	0.50	0.50
Vitamin-mineral premix[2]	0.25	0.25	0.25
Antibiotic[3]	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
Calcium	0.90	0.85	0.70
Phosphorus	0.75	0.70	0.60

[1] As-fed basis.
[2] Provided the following per kilogram of complete diet: vitamin A as retinyl acetate, 5,510 IU; vitamin D_3, 1,102 IU; vitamin E as dl-α-tocopherol, 22 IU; vitamin B_{12}, 0.022 mg; riboflavin, 4.4 mg; niacin as niacinamide, 22 mg; d-pantothenic acid as dl-calcium pantothenate, 17.6 mg; vitamin K as menadione dimethylpyrimidinol bisulphite, 4.4 mg; choline as choline chloride, 220 mg; folic acid, .33 mg; thiamin as thiamine 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.
[3] Supplied 55 mg carbadox/kg of complete diet.

Zn supplementation of nursery and grower diets on performance and immune responses of grower pigs inoculated with PRRSv and *M. hyopneumoniae*.

MATERIALS AND METHODS

General procedures

Experimental protocols used in this study were approved by the North Carolina State University Institutional Animal Care and Use Committee. Seventy-two crossbred pigs (York/Landrace×Hampshire) were weaned at 21 d of age (average initial BW 7.0±0.02 kg) and assigned by weight, regardless of sex, to one of eight treatment groups (n=9 pigs/group). Pigs were housed 9 pigs per pen in the nursery for 6 wk, and then moved to an off-site curtain sided finishing barn. The sow farm and nursery are PRRSv-free facilities as described previously (Roberts and Almond, 2002). There were no other pigs in the finishing facility at the time of the study and the control and treatment groups were housed in separate rooms at opposite ends of the facility. Within treatment (n=9 pigs/treatment), pigs were housed in groups of similar weight; three pigs per pen (5.5 m^2) in the finishing facility. A 2×4 factorial arrangement of treatments was used in a randomized design to determine the effects of dietary Zn and PRRSv and *M. hyopneumoniae* infection. Factors included; 1) intranasal administration of PRRSv and intra-tracheal infusion of *M. hyopneumoniae*, or sham inoculation with vehicle; 2) supplemental Zn at 10, 50, 150 ppm or 2,000 ppm. Diets were fed for 6 wk in the nursery and continued into grow/finish for 31 d. Thus, pigs assigned to the 10, 50 or 150 ppm supplemental Zn groups received the diet throughout the study. The diet with 2,000 ppm supplemental Zn was fed for the first 2 wk in the nursery and then pigs received a diet with 150 ppm supplemental Zn for the remainder of the trial.

Entry into the grow/finish facility was designated d 0. At this time, pigs were inoculated with PRRSv and *M. hyopneumoniae* or sham-inoculated with media (control). Feed and water were supplied *ad libitum*. Pigs were weighed at weaning, prior to inoculation at d 0, and at d 10, 17, 24 and 31. Weekly feed intake was determined for 31 d in the grow/finish facilities and blood samples were collected weekly. The blood samples were used for quantification of alkaline phosphatase (ALP) and serum Zn concentrations. General animal health was noted daily by monitoring attitude, clinical appearance, and behavior. Rectal temperatures were obtained from one pig per pen at d 0 and daily thereafter for 28 d.

Diets

Experimental diets (Table 1) were formulated in accordance to nutrient estimates provided by the National Research Council (NRC, 1998). The notable exception to the guidelines was dietary Zn. Zinc was supplemented as $ZnSO_4$ to a corn-soybean-meal based diet containing 34 ppm Zn. The levels were chosen to reflect Zn levels below (10 ppm) and at the NRC minimum level of 60 ppm (50 ppm), grow/finish supplementation at three times NRC minimum level (150 ppm), and to evaluate the industry practice of nursery supplementation at pharmacological levels (2,000 ppm). Analyzed Zn concentrations in the experimental diets were 41.3, 84.0, 177.4 and 2,061.6 for the prestarter diets, 56.7, 86.6, and 187.4 for the starter diets, and 48.2, 83.6 and 198.5 for the grower diets. Dietary Zn and serum Zn concentrations were confirmed by atomic absorption spectrophotometry (model AA6701F Shimadzu, Norcross, GA). Serum ALP activity was measured using Sigma Diagnostics Alkaline Phosphatase reagent® (ALP 50, Sigma Chemicals, St. Louis, MO), which measures ALP activity by a kinetic method similar to the procedure described by Bowers and McComb (1966).

Pathogen preparation, inoculation and assessment

The PRRSv inoculum was prepared as previously described (Roberts and Almond, 2002). Pigs in the treatment group were inoculated by intranasal administration of 1 ml of PRRSv (10^{3-4} $TCID_{50}$, isolate SD

23,983; Rossow et al., 1994). Uninfected media was used for the inoculations in the control group. PRRSv isolation, as described by Stevenson et al. (1994), was conducted on serum samples collected on d 0 and 28. Briefly, 100 µl of sera was added to cultured alveolar macrophages and allowed to incubate for 3 to 5 days and then read for cytopathic effects. Cultures were deemed positive if there was any indication of cell death. An ELISA (Herd Check®; IDEXX Laboratories, Inc., Westbrook ME) for PRRSv was used to detect the presence of antibody to PRRSv in serum samples from d 0 and d 28 to confirm inoculation and to verify the virus free status of the control group.

The *M. hyopneumoniae* inoculum (LI31 5-13-93, Strain 11) was obtained from Drs. E. Thacker and R. Ross at Iowa State University, Ames, Iowa. The *M. hyopneumoniae* inoculum consisted of a 10% lung suspension of 4 parts *M. hyopneumoniae* strain 11 and 1 part 24 h low-passage broth culture of the same strain. Pigs in the treatment group were inoculated by intra-tracheal infusion of 5 ml *M. hyopneumoniae* (10^6 organisms/ml). Intra-tracheal infusion was performed as previously described (Ross and Cox, 1988; Roberts and Almond, 2002). Uninfected cell cultures were used as vehicle inoculations for the control group. For the serological assessment of *M. hyopneumoniae,* serum samples were tested with an ELISA (DAKO *Mycoplasma hyopneumoniae* ELISA®; DAKO A/S, Glostrup, Denmark) on samples collected at d 0 and d 28.

Immune response measurements

Immune responses were assessed using previously established methods (van Heugten et al., 1994b; Roberts et al., 2002). Cellular immune response was measured *in vivo* on d 13 using a phytohemagglutinin (PHA) skin test (Kornegay et al., 1989). One randomly selected pig per pen was injected subcutaneously in the right flank fold with 0.1 ml of PHA (150 µg/ml; Sigma Chemicals). Skin fold thickness was determined at 0, 6, 12, 24 and 48 h post injection of PHA. The *in vitro* cellular immune response, as determined using a lymphocyte blastogenesis assay (Blecha et al., 1983), was measured on d 20 in one pig per pen randomly selected from one of the two pigs per pen not receiving the PHA skin test. Approximately 15 ml of blood was collected by venipuncture into heparinized tubes. 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 PHA and pokeweed mitogen (PWM, Sigma Chemical) were used as mitogens at concentrations of 10 µg/ml each. These mitogen concentrations were shown to provide near maximum stimulation of blood mononuclear cells (van Heugten et al., 1994a). Cells were incubated at 37°C in 5% CO_2 atmosphere for 48 h. Cultures were then pulsed with ^{3}H-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 ^{3}H-thymidine served as the measure of cell proliferation.

To determine primary humoral immune response, the remaining pig, not subjected to immune response measurements, was injected i.m. with 1 ml of a 20% suspension of sheep red blood cells (SRBC) in phosphate buffered saline (PBS), 7 d after PRRSv inoculation. Blood samples were taken at the time of SRBC injection, and 7 and 14 d after injection for determination of total immunoglobulin (Ig), IgG and IgM titers to SRBC. Titers were measured by a microtiter hemagglutination assay (Wegmann and Smithies, 1966) with modifications (van Heugten et al., 1994a). Titers were recorded as $\log_2$ of the reciprocal of the highest dilution that caused agglutination of SRBC.

Statistical analyses

Data were analyzed as a randomized design with a 2×4 factorial arrangement of treatments using the GLM procedure of SAS (1988). The model included disease challenge, Zn, and the disease challenge×Zn interaction. Pen means were used to analyze pig performance, whereas individual pig data served as the experimental unit in the immune response data. Initial skin thickness, Zn and ALP concentrations were used as covariates in the analyses of PHA skin thickness response, and serum Zn and ALP concentrations, respectively. Significance of differences between treatments was determined by using the least significant difference method. Least squares means are reported. Differences in rectal temperatures were analyzed by repeated measures.

RESULTS AND DISCUSSION

Stimulation of the immune system during a disease challenge may result in the partitioning of dietary nutrients away from growth in favor of metabolic processes that support the immune response and resistance to disease. This forms the basis for impaired growth and feed utilization, and altered nutrient requirements (Klasing and Johnstone, 1991). Disease interactions seen (or suspected) under field conditions largely have not been reproduced experimentally (Pijoan, 1996). In the present study, pigs were inoculated with PRRSv and *M. hyopneumoniae* using a previously established disease model (Roberts and Almond, 2002). *Mycoplasma hyopneumoniae* is a causative agent of porcine enzootic pneumonia, a mild, chronic pneumonia commonly complicated by opportunistic infections with other bacteria (Kobisch and Friis, 1996). In contrast, PRRSv induces a severe, acute pneumonia with clinical disease characterized

Table 2. Results of diagnostic tests for PRRSv and *M. hyopneumoniae* (M. hyo). The P- and P+ refer to the PRRSv inoculation status of a group

Day	Diagnostic test							
	PRRSv isolation[1]		PRRSv ELISA				M. hyo ELISA[a]	
			S/P ratio[2]		Range-S/P[3]			
	P-	P+	P-	P+	P-	P+	P-	P+
d 0	0/36	0/36	0.00	0.01	0-0.03	0-0.112	3/36	4/36
d 28	0/36	30/36	0.02	2.28	0-0.24	1.31-3.09	12/36	19/36

[1] Total number of pigs positive/total number pigs per treatment group.
[2] Each value represents the mean of 36 pigs. Ratios of <0.40 were considered negative for PRRSv (Benfield et al., 1999). The S/P is the sample/positive control ratio for the ELISA test.
[3] Minimum and maximum range of S/P group.

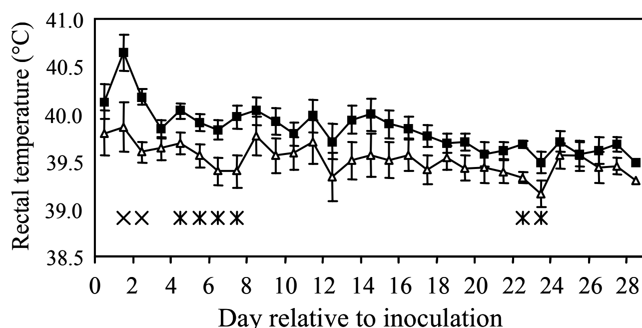

Figure 1. Effects of PRRSv on rectal temperature in pigs with natural exposure to *M. hyopneumoniae* (Δ) and pigs exposed to both PRRSv and *M.* hyopneumoniae (■). Rectal temperatures differed (×, p<0.10; *, p<0.05) between groups for d 1, 2, 4, 5, 6, 7, 22 and 23.

by labored, abdominal respiration and tachypnea (Rossow, 1998). Recently, a respiratory syndrome, designated porcine respiratory disease complex (PRDC), has emerged as a serious health problem in swine. PRRSv and *M. hyopneumoniae* are two common pathogens isolated from pigs exhibiting PRDC (Thacker et al., 1999). Therefore, the present study used PRRSv and *M. hyopneumoniae* to simulate disease situations found in commercial farms.

In the initial experimental design, the treatment group was inoculated with *M. hyopneumoniae*. However, animals in the treatment and control groups had antibodies to *M. hyopneumoniae* prior to the experimental inoculation (Table 2). By d 28, 12 animals in the control group had antibodies to *M. hyopneumoniae,* thereby confirming prior exposure and spread of the organism to other pigs. Therefore, both groups of pigs had natural exposure to *M. hyopneumoniae* and the experimental inoculation had minimal influence on the number of pigs with detectable antibodies to the organism. Compared with our previous study (Roberts and Almond, 2002) with pigs from the same farm and age, these observations were unexpected. Thus, the only health difference between the control and treatment groups was PRRSv infection. Therefore, the results are presented to reflect this difference in disease status.

Pigs in the PRRSv group exhibited clinical signs (dyspnea, lethargy, increased rectal temperatures, and anorexia) consistent with PRRSv infection by 3 days post inoculation. Coughing was noted by d 10 in most animals of the PRRSv group. In control pigs, coughing was intermittent without a discernible relation to the initiation of the study. Based on subjective daily observations, inoculation of pigs with *M. hyopneumoniae* and PRRSv increased the severity and duration of respiratory clinical signs, including coughing, compared to pigs exposed to *M. hyopneumoniae* alone. This finding concurs with a report that indicated inoculation with *M. hyopneumoniae* and PRRSv induced more severe clinical respiratory disease than single organism infected groups (Thacker et al., 1999). Rectal temperatures differed between PRRSv inoculated pigs and control pigs from d 4 to 7 and d 22 and 23 (Figure 1). The time frame is consistent with published reports for PRRSv (Zimmerman et al., 1997).

Average daily gain, feed intake and gain:feed did not differ among the four supplemental Zn levels (Table 3). This failure to induce changes in gain and feed intake concurs with a previous study, which examined the effect of dietary Zn levels in 30 kg BW gilts (Hill and Miller, 1983). Pigs inoculated with PRRSv had lower feed intake (p<0.01) than the control pigs for most of the 31 d study (Table 3). However, gain differed (p<0.01) from d 10-17 and tended (p<0.10) to differ for the 31 d. Few differences in gain:feed were observed with the exception of d 24-31 when the PRRSv inoculated pigs had greater (p<0.01) gain:feed than control pigs.

The reduced weight gain and feed intake could be attributed to the anorexia associated with the PRRSv infection. However, changes in metabolism resulting from immune activation may also contribute to differences in weight gain. Increased basal metabolic rate and decreased muscle accretion due to increased rates of protein degradation and decreased protein synthesis reportedly occur during the homeorhetic response (Klasing and Johnstone, 1991; Spurlock, 1997). Interestingly, on d 24 to 31 feed intake and gain did not differ, while gain:feed was greater (p<0.01) for PRRSv and *M hyopneumoniae* inoculated pigs compared to gain:feed of the control pigs. The improved feed conversion in pathogen-inoculated pigs may reflect a compensatory response in order to recover

Table 3. Effect of PRRSv and dietary Zn on gain, feed intake, and efficiency of gain of finishing pigs[1]

Item	PRRSv status		SEM	Supplemental $ZnSO_4$ (ppm)				SEM
	P-	P+		10	50	150	2,000	
Gain, kg/d								
d 0-10	0.66	0.61	0.10	0.62	0.59	0.66	0.67	0.14
d 10-17[b]	1.04	0.66	0.18	0.75	0.98	0.91	0.76	0.29
d 17-24	0.85	0.69	0.16	0.80	0.71	0.83	0.74	0.22
d 24-31	0.87	0.96	0.11	0.92	0.86	0.88	1.00	0.15
d 0-31[a]	0.84	0.72	0.07	0.76	0.76	0.80	0.78	0.10
Feed intake, kg/d								
d 0-10[a]	1.31	1.16	0.13	1.27	1.12	1.28	1.27	0.18
d 10-17[b]	1.75	1.29	0.18	1.49	1.47	1.63	1.49	0.25
d 17-24[b]	1.98	1.56	0.19	1.75	1.75	1.84	1.75	0.26
d 24-31	2.10	1.91	0.22	2.02	1.97	2.03	2.00	0.30
d 0-31[b]	1.74	1.45	0.16	1.60	1.53	1.65	1.60	0.22
Gain:feed								
d 0-10	0.51	0.53	0.03	0.48	0.54	0.52	0.53	0.05
d 10-17	0.60	0.52	0.05	0.51	0.66	0.56	0.51	0.06
d 17-24	0.43	0.44	0.03	0.46	0.41	0.45	0.42	0.05
d 24-31[b]	0.42	0.50	0.02	0.46	0.44	0.44	0.50	0.03
d 0-31[a]	0.48	0.50	0.01	0.48	0.50	0.49	0.49	0.01

[1] Data for the PRRSv status and $ZnSO_4$ supplementation of nursery diets are means of 12 pens of three pigs and six pens of three pigs, respectively. Diets with 10, 50 and 50 ppm supplemental $ZnSO_4$ were provided from weaning until the end of the study. The diet with 2,000 ppm $ZnSO_4$ was provided for the first two weeks after weaning, and then pigs received a diet with 150 ppm supplemental $ZnSO_4$. Pigs in the P+ group were inoculated with PRRSv and *M. hyopneumoniae*. The P- pigs were not inoculated and were intended to serve as controls; however, by d 28, similar numbers of pigs in both groups were serologically positive for *M. hyopneumoniae.*
[a] Disease effect (p<0.10). [b] Disease effect (p<0.01).

Table 4. Effect of PRRSv and dietary Zn on serum Zn concentrations (ppm)[1]

Day[2]	PRRSv status		SEM	Supplemental $ZnSO_4$ (ppm)				SEM
	P-	P+		10	50	150	2,000	
d 0	1.34	1.19	0.04	1.22	1.23	1.35	1.23	0.07
d 7[ab]	1.32	1.14	0.05	1.08[d]	1.14[de]	1.50[f]	1.21[e]	0.07
d 14[ac]	1.69	1.48	0.06	1.38[d]	1.68[e]	1.61[de]	1.67[e]	0.09
d 21[b]	1.44	1.50	0.07	1.35[de]	1.23[d]	1.76[f]	1.53[ef]	0.10
d 28[b]	1.37	1.31	0.06	1.01[d]	1.30[e]	1.58[f]	1.47[ef]	0.08

[1] Initial serum Zn concentration was used as a covariate. Values represent the means of 12 pens (3 pigs/pen) and 6 pens (3 pigs/pen) for PRRSv status and supplemental $ZnSO_4$, respectively. [2] Days relative to PRRSv inoculation.
[a] Disease effect (p<0.05). [b] Zn effect (p<0.01). [c] Zn effect (p<0.05).
[d, e, f] Within $ZnSO_4$ level, means within row lacking a common superscript differ (p<0.05).

from the previous three weeks of depressed gain. It is noteworthy that the feed intake by inoculated pigs was similar (p=0.20) to control pigs during d 24 to 31, whereas feed intake previously differed between groups. Our data suggests that under favorable environmental and husbandry conditions, pigs recovered from the experimental pathogen challenge. Conversely, the particular strain of PRRSv may have induced a mild disease, which was not sufficiently severe to induce long-term effects on growth.

A Zn effect (p<0.05) was evident on d 7 to 28 and serum Zn concentrations tended to increase with increasing Zn supplementation (Table 4). However, few differences were noted in the groups receiving a diet with 150 ppm supplemental Zn and groups fed 2,000 ppm supplemental Zn for two weeks in the nursery and then 150 ppm for the remainder of the study. A disease effect (p<0.05) was identified at d 7 and 14. In general, serum Zn concentrations were similar to previous reports (Liptrap et al., 1970; Roberts et al., 2002).

Serum ALP concentrations differed (p<0.05) between control pigs and the PRRSv inoculated pigs at d 7, 14 and 21 (Table 5). The ALP concentrations were less (p<0.05) in the group of pigs supplemented with 10 ppm Zn than the other groups at d 14 and 21. Serum ALP concentrations were similar among the 50, 150 and 2,000 ppm Zn supplementation groups. It was reported that a threshold is reached between 50 and 500 ppm in which the Zn fails to stimulate ALP activity (Hill and Miller 1983).

As dietary Zn concentration increases, intestinal metallothionein also increases and the excess Zn-metallothionein complexes are not absorbed (Carlson et al., 1999), thereby regulating Zn homeostasis. The disease effect on serum ALP and Zn concentrations may be explained by previous observations that immune challenge causes redistribution of Zn within the body due to the hepatic synthesis of metallothionein (Klasing and Johnstone,

Table 5. Effect of PRRSv and dietary Zn on serum alkaline phosphatase concentrations (U/L)[1]

Day[2]	PRRSv status		SEM	Supplemental $ZnSO_4$ (ppm)				SEM
	P-	P+		10	50	150	2,000	
d 0	194.1	201.0	8.1	181.8	209.5	216.9	182.1	11.5
d 7[a]	198.5	160.7	10.5	153.3	201.7	188.9	174.5	15.0
d 14[ab]	183.7	159.0	8.8	140.1[d]	177.9[e]	190.4[e]	177.1[e]	12.6
d 21[ac]	190.9	168.3	10.9	150.7[d]	176.1[de]	198.0f[e]	193.7[e]	15.7
d 28	173.5	181.9	12.9	161.6	177.3	209.1	162.8	18.5

[1] Initial serum ALP concentration was used as a covariate. Values represent the means of 12 pens (3 pigs/pen) and 6 pens (3 pigs/pen) for PRRSv status and supplemental $ZnSO_4$, respectively. [2] Days relative to PRRSv inoculation.

[a] Disease effect ($p<0.05$). [b] Zn effect ($p<0.05$). [c] Zn effect ($p<0.10$).

[d, e] Means within the main effect of $ZnSO_4$ supplementation and within row lacking a common superscript differ ($p<0.05$).

Table 6. Effect of PRRSv and dietary Zn on change in skin thickness response to phytohemagglutinin (PHA)[1]

Item	PRRSv -				SEM	PRRSv+				SEM
added $ZnSO_4$, ppm	10	50	150	2,000		10	50	150	2,000	
Skin thickness response, cm										
6 h	0.48	0.80	0.78	0.57	0.08	0.52	0.62	0.88	0.57	0.08
12 h	0.75	1.13	0.95	1.07	0.08	0.97	1.20	0.98	0.73	0.10
24 h[ab]	0.65[cd]	1.00[c]	0.20[e]	0.80[cd]	0.17	0.42[de]	0.58[de]	0.62[cd]	0.57[de]	0.04
48 h	0.30	0.25	0.20	0.33	0.03	0.17	0.28	0.22	0.17	0.03

[1] Initial skin thickness was used as a covariate. Each value represents the mean of three pigs. Pigs were injected with PHA at d 13 of the study.

[a] Zn effect ($p<0.10$). [b] Disease×Zn interaction ($p<0.05$). [c, d, e] Means within row lacking a common superscript differ ($p<0.05$).

1991). It was proposed that metallothionein binds Zn and thus reduces the quantity of Zn in circulating pools (Carlson et al., 1999).

Few differences in *in vivo* cellular immune response, as measured on d 13 by skin thickness response to PHA, were evident with the exception of a Zn effect ($p<0.10$) at 24 h (Table 6). The *in vitro* cell-mediated immune response was measured in one pig per pen on d 20, using a lymphocyte blastogenesis assay. Neither supplemental Zn nor PRRSv affected *in vitro* lymphocyte proliferation. Whereas unstimulated lymphocyte response was 52.5±9.8 cpm×10^3, lymphocyte responses to PHA were 115.3±11.9 and 116.6±12.5 cpm×10^3 in control and PRRSv inoculated groups, respectively. For Zn groups, lymphocyte response was 117±17.6 cpm×10^3. The responses to PWM also were not affected ($p>0.20$) by Zn or PRRSv inoculation (data not shown).

It is recognized that PRRSv modulates or alters respiratory and systemic immune responses (Thacker, 2001). Cell-mediated immune mechanisms are important in *M. hyopneumoniae* infections (Tajima et al., 1984), and dual infections with *M. hyopneumoniae* and PRRSv induced greater and more consistent *in vitro* expression of proinflammatory cytokines than infection with either pathogen (Thanawongnuwech et al., 2001). Thus, we anticipated differences in the PHA skin test and lymphocyte proliferation responses. The failure to detect differences between PRRSv inoculated pigs and control pigs may be related to the timing of the immune response tests. Proliferative lymphocyte and T_H1-cell mediated immune responses to PRRSv were first detected at four weeks post-infection (Bautista and Molitor, 1997; Rossow, 1998).

Although cell-mediated immune mechanisms are important in *M. hyopneumoniae* infections, similar numbers of seropositive pigs were detected in the control and PRRSv groups at d 28 after inoculation. Consequently, it is unlikely that *M. hyopneumoniae* played a role in the failure to observe differences in the immunological tests. Conversely, the *M. hyopneumoniae* may have compromised the immune responses (Tajima et al., 1984; Thacker 2001). The present results indicate that Zn supplementation had minimal effects on cell-mediated immune responses. This observation conflicts with previous studies that reported a stimulatory effect of Zn on human T-cells (Driessen et al., 1994) and conversely, inhibition of T-cell proliferation (Wellinghausen et al., 1999) and PHA stimulation of lymphocytes (Chandra, 1984) by high Zn concentrations. However, the present results concur with a prior study that showed dietary Zn levels failed to affect *in vitro* mitogen stimulation of lymphocytes and intradermal response to PHA in finishing steers (Spears and Kegley, 2002).

Total Ig and IgG responses to SRBC (Table 7) differed ($p<0.10$) on d 0 (which corresponds to d 7 after virus inoculation) for the four Zn levels. Presumably, stimulation of humoral immunity must have occurred prior to the SRBC immunization. With the same numbers of PRRSv-inoculated pigs assigned to each Zn level and the absence of a disease effect at d 0, the differences cannot be attributed to PRRSv infection. On d 7, control pigs had increased levels of IgG ($p<0.05$) compared with pigs inoculated with PRRSv. IgM antibody response to SRBC (Table 7) was greater ($p<0.05$) for PRRSv inoculated pigs compared to control pigs on d 14. While the humoral response is important in host defense against both PRRSv and *M*

Table 7. Effects of PRRSv and dietary Zn on total immunoglobulin (Ig) response, IgG and IgM to intramuscular injection with sheep red blood cells (SRBC)[1, 2]

Item	PRRSv status		SEM	Supplemental $ZnSO_4$ (ppm)				SEM
	P-	P+		10	50	150	2,000	
Ig, HA units								
d 0[3,a]	1.25	1.58	0.16	1.00[c]	1.50[cd]	2.00[d]	1.17[c]	0.23
d 7	1.83	1.50	0.16	1.33	1.67	1.83	1.83	0.23
d 14	2.08	2.33	0.16	2.00	2.00	2.33	2.50	0.23
IgG, HA units								
d 0[a]	1.17	1.33	0.12	1.00[c]	1.17[c]	1.67[d]	1.17[c]	0.17
d 7[b]	1.83	1.33	0.03	1.33	1.67	1.67	1.67	0.20
d 14	2.00	1.75	0.16	1.67	1.83	1.83	2.17	0.22
IgM, HA units								
d 0	0.08	0.25	0.11	0	0.33	0.33	0	0.15
d 7	0	0.17	0.11	0	0	0.17	0.17	0.15
d 14[b]	0.08	0.58	0.11	0.33	0.17	0.50	0.33	0.15

[1] One pig per pen (n=6/ZSO_4 treatment; n=12/PRRSv status) was injected with SRBC on d 14 of the study. Pigs were injected with SRBC on d 7 after the PRRSv inoculations.

[2] Values are expressed as $\log_2$ of the reciprocal of the highest dilution causing agglutination (HA titers) of SRBC.

[3] Indicates days after SRBC injection. Days 0, 7 and 14 correspond to d 7, 14 and 21 after PRRSv inoculation.

[a] Zn effect (p<0.10). [b] Disease effect (p<0.05).

[c, d] Means within the main effect of $ZnSO_4$ supplementation and within row lacking a common superscript differ (p<0.05).

hyopneumoniae, synergy between PRRSv and *M. hyopneumoniae* may result in antibody responses that contribute to disease pathogenesis through delays or decreases in Ig concentrations. Simecka et al. (1993) reported that mycoplasmal antibody responses might contribute to disease pathogenesis through the development of hypersensitivity responses or through the deposition of immune complexes. In addition, polyclonal activation of B cells and an exaggerated humoral immune response occur in lymphoid organs from PRRSv-infected pigs (Lamontagne et al., 2001). Since d 7 of the SRBC immunization procedure corresponds to d 14 after PRRSv inoculation, it is possible that the humoral response to PRRSv interfered with the IgG response to SRBC. Conversely, the early polyclonal activation of B cells in PRRSv-infected animals may exaggerate the IgM response, and thus explain the enhanced IgM response to SRBC at d 14. The precise role of Zn in modulating humoral responses currently is poorly understood. However, results of the present study indicate that supplemental dietary Zn had minimal influence on humoral response to SRBC. This observation agrees with previous studies of Zn supplementation in weaned pigs (Cheng et al., 1998) and finishing steers (Spears and Hegley, 2002).

IMPLICATIONS

In the present study, PRRSv and *M. hyopneumoniae* inoculation acted to impair performance of pigs. It is debatable whether the pathogens acted synergistically. However, serological results indicated that an initial exposure to *M. hyopneumoniae* commenced in both groups prior to the study. Our results concur with previous reports that infection with *M. hyopneumoniae* does not impair performance to the same extent as a co-infection with PRRSv and *M. hyopneumoniae*. Furthermore, Zn supplementation failed to mitigate the performance deficits caused by co-infection with these pathogens. Under our experimental conditions of excellent management, hygiene, adequate floor space and unrestricted feed allowance, compensatory gain resulted in trial ending weights that did not differ between groups. These results indicate that there are few, if any, benefits gained by Zn supplementation to grower pigs exposed to PRRSv and *M. hyopneumoniae*. Conventional immune measurements demonstrated the complexity of the interaction between dietary Zn, and PRRSv and *M. hyopneumoniae* infections.

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