Influence of Level and Source (Inorganic vs Organic) of Zinc Supplementation on Immune Function in Growing Lambs*

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ABSTRACT : Eighteen lambs were used to determine the effects of zinc (Zn) level and source on Zn status and immune function during both normal conditions and conditions of physiologic stress. Treatments consisted of a basal diet (27.6 mg of Zn/kg), and the basal diet supplemented with 25 mg of Zn/kg, added as either zinc oxide or zinc methionine. The basal diet was a corn-cottonseed hull-isolated soy protein- based diet (14% CP). Lambs were weighed and blood samples taken at 28-d intervals for determination of serum Zn and alkaline phosphatase activity. Weights and serum Zn were similar ($p > 0.10$) among treatments at all sampling days. To evaluate immune responses and Zn status during conditions of physiologic stress lambs were administered 100 I.U. of adrenocorticotrophin (ACTH) on d 112 and feed was withheld for 48 h.

Cortisol levels were elevated ($p < .01$) 5 h post ACTH injection, but had returned to initial levels after 48 h. Lymphocyte blastogenesis ([^{3}H]-thymidine incorporation) on d 112 (prior to ACTH injection) and 114 was unaffected ($p > .10$) by dietary treatment. However, blastogenesis in response to pokeweed mitogen was greater ($p < .0001$), whereas the response to phytohemagglutinin was reduced ($p < .01$) following ACTH administration and fasting. Antibody response to administration of porcine red blood cells was unaffected ($p > .05$) by dietary treatment. These results indicate that, given the Zn concentration of the basal diet, there was no enhancement of immune function by supplemental Zn, either before or after lambs were subjected to stress.

(Key Words: Zinc, Zinc Methionine, Zinc Oxide, Immune Function, Lambs)

INTRODUCTION

Previous research demonstrated that severe zinc (Zn) deficiency in lambs decreased *in vitro* phytohemagglutinin (PHA)-induced and increased pokeweed mitogen (PWM)-induced lymphocyte blastogenesis (Droke and Spears, 1993). Marginally deficient lambs had immune responses and weight gains similar to Zn adequate lambs. A semi-purified diet was used in the previous study and in contrast little is known about the effect of Zn supplementation on immune function in lambs consuming practical diets marginally deficient in Zn.

Diets for domestic livestock are usually supplemented with Zn in the inorganic form as either zinc oxide (ZnO) or zinc sulfate ($ZnSO_4$). Zinc methionine (ZnMet) is an organic Zn complex which has improved performance (Spears, 1989; 1996) and carcass characteristics of beef cattle (Greene et al., 1988). Zinc methionine also may improve immune function and disease resistance in stressed animals. Stressed steers supplemented with ZnMet had greater antibody titers against bovine herpes virus-1 following vaccination than steers fed a similar level of Zn from ZnO (Spears et al., 1991). In lambs stressed by weaning and shipping, ZnMet and ZnO supplementation appeared to effect cellular immunity differently (Kegley and Spears, 1995).

Supplementation of practical diets marginally deficient in Zn may improve immune function, and since Zn from ZnMet appears to be metabolized differently than ZnO (Spears, 1989), ZnMet may affect immune responses differently than inorganic Zn sources. The objective of the present study was to determine the effect of Zn level and source on immune response of lambs fed practical diets marginal in Zn.

MATERIALS AND METHODS

* Use of trade names in this publication does not imply endoresment by the North Carolina ARS or criticism of similar products not mentioned.

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Animals and diets

Eighteen crossbred lambs averaging 18.4 ± 0.6 kg were used in this study. Animal care was in compliance with applicable guidelines from the North Carolina State University Policy on Animal Care and Use. Dietary treatments consisted of: 1) basal diet, 2) basal diet supplemented with 25 mg of Zn/kg as ZnO (feed grade), and 3) basal diet supplemented with 25 mg of Zn/kg as ZnMet (ZinPro 100, Zinpro, Edina, MN).

Table 1. Composition of basal diet

Ingredient	% of DM
Cottonseed hulls	30.00
Soy protein	7.50
Corn	60.60
Limestone	1.42
Salt	.50
Vitamin Mix[a]	.03
Mineral Mix[b]	.01

[a] Provided in mg/kg of vitamin mix: retinyl acetate, 3,407; cholecalciferol, 82.5; and, all-*rac*-α-tocopherol, 3,301.
[b] Provided in mg/kg of diet: MnO, 67; KI, 0.1; $CoCl_2 \cdot 6H_2O$, 0.4; Na_2SeO_3, 0.22; $CuSO_4$, 15.9.

The basal diet (table 1) contained 89.3% dry matter and 27.6 mg of Zn/kg, and was formulated to meet NRC (1985) requirements for growing lambs, with the exception of Zn. The level of supplemental Zn used in treatments 2 and 3 was chosen based on NRC (1985) requirements for growing lambs. Including the Zn in the basal diet, the level of supplemental Zn added should have met but not greatly exceeded Zn requirements. Lambs were grouped by weight and randomly assigned to treatment within groups (n = 6).

Lambs were individually housed in 1.52m^2 plastic pens equipped with plastic feeders, waters and plastic-coated slotted floors in a temperature-controlled room (25℃). Lambs had free access to deionized water throughout the study and were fed *ad libitum*. Feed was offered *ad libitum* the first 44 d of the study. From d 45 until the end of the study each lamb was fed 1,100 g/d. Prior to feeding, body weights and blood samples via jugular venipuncture were taken at 28 d intervals for determination of serum Zn and alkaline phosphatase (ALP) activity. These measurements were used as indicators of Zn status (Apgar, 1979).

Stress simulation and sample collection

To evaluate the effect of Zn supplementation on immune function and Zn status (serum Zn) during conditions of physiologic stress, on d 112, lambs were administered 100 I.U. ACTH (porcine ACTH; Sigma Chemical, St. Louis, MO) intramuscularly and feed was withheld for 48 h. The following samples were collected at the designated times: serum Zn (0, 5 and 48 h, 7 and 14 d), cortisol (0, 5 and 48 h) and peripheral blood mononuclear cells for blastogenesis assays (pre-ACTH and fasting on d 112 and 48 h later). Forty-eight h after ACTH administratin and initiation of fasting, lambs were injected with porcine red blood cells (RBCs) to measure the humoral immune response.

The level of ACTH administered was based on a preliminary dose titration study where serum cortisol concentrations were measured following ACTH injection.

Analytical procedures

Feed samples were prepared for Zn analysis by wet ashing using nitric acid and hydrogen peroxide in a microwave digester (Model MDS-81D, CEM, Matthews, NC). Zinc concentrations of the basal diet and serum samples were determined by atomic absorption spectrophotometry (Model 5000, Perkin Elmer, Norwalk, CT). Serum cortisol concentrations were determined using a solid-phase radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Hemagglutination titers to porcine RBCs were determined using the procedure of Droke et al. (1993). *In vitro* mitogen-induced lymphocyte blastogenesis was measured using the ^{3}H-thymidine incorporation procedure of Droke and Spears (1993). Three mitogens (Sigma Chemical, St. Louis, MO) were utilized: phytohemagglutinin (PHA), pokeweed mitogen (PWM) and lipopolysaccharide (LPS, *E. coli* O11:B4). Two sources of serum were used in the blastogenesis assays: autologous serum (AS, collected from each lamb prior to the assay) and fetal calf serum (FCS) which is traditionally used in this type of assay. Alkaline phosphatase activity was measured colorometrically (Sigma, 1987).

Statistical analyses

Statistical analysis of data was performed by analysis of variance using the GLM procedures of SAS (1985). The model for feed intake, weight gain, and serum Zn and ALP (prior to ACTH and fasting) included treatment. Lymphocyte blastogenesis data were ln transformed for statistical analysis. The data are presented as the ln transformed means with the back transformed means given in paratheses. The model for lymphocyte blastogenesis included treatment, lamb within treatment, serum, day, and interactions among treatment, serum and day. Tukey's studentized range test was used for mean comparisons when interactions were significant. Serum Zn,

ALP and cortisol data during the period of ACTH and fasting were analyzed using a model that included treatment, lamb within treatment, time, and treatment by time interaction. The effect of treatment was tested using the lamb within treatment mean square error as the error term for treatment.

RESULTS AND DISCUSSION

Zinc supplementation, either as ZnO or ZnMet, did not result ($p > .05$) in improved growth when compared to the basal diet (table 2). This indicates the Zn concentration of the basal diet (27.6 mg of Zn/kg) was sufficient to support growth. Feed intake also was not affected by treatment (data not shown). Depressed feed intakes and weight gains (Droke and Spears, 1993; Apgar and Fitzgerald, 1985) have been observed in sheep with clinical signs of Zn deficiency (diets contained 4 and < 1 mg of Zn/kg); however, lambs fed marginally Zn deficient diets (6-9 mg of Zn/kg, Droke and Spears, 1993; Droke et al., 1993) exhibited no signs of Zn deficiency and had similar feed intakes and weight gains to Zn adequate lambs. The previous studies utilized semi-purified diets and, generally, practical sheep diets will not have dietary Zn concentrations this low. A severe Zn deficiency is seldom observed under practical conditions and the Zn requirement for sheep ranges from 20-33 mg of Zn/kg of diet (NRC, 1985). The supplementation of Zn as an organic complex (ZnMet) also did not improve growth over the inorganic source (ZnO). This is in contrast to the improved performance observed with heifers (Spears, 1989). Heifers fed a corn silage-based diet containing 24 mg of Zn/kg had similar weight gains and feed efficiency to heifers fed the diet supplemented with 25 mg of Zn/kg as ZnO (Spears, 1989). Supplementation with 25 mg of Zn/kg as ZnMet resulted in faster and more efficient gains. The lack of improved performance in the present study may reflect species differences in the metabolism and utilization of ZnMet or the small number of animals used.

Table 2. Effect of level and source of zinc on body weight gain of lambs

Item	Treatment			SEM
	Control	ZnO	ZnMet	
	..... ADG, kg/d			
d 0- 28	0.17	0.15	0.14	0.02
d 29- 57	0.21	0.21	0.19	0.01
d 58- 84	0.19	0.19	0.17	0.01
d 85-112	0.19	0.19	0.18	0.01

Serum ALP activity and Zn concentrations (table 3) were unaffected ($p > .05$) by dietary treatment. Serum ALP activities in lambs fed the basal and ZnO diets tended to decrease over the duration of the study, whereas lambs fed the ZnMet diet maintained fairly constant levels of serum ALP activity from d 28 to d 112. Zinc supplementation (as ZnMet) resulted in greater ($p < .05$) serum ALP activities at the end of the study (d 112). This may reflect a difference in Zn status of the lambs, even though no dufferences were observed in weight gain or feed intake. However, there is no good measure of marginal Zn deficiency for livestock, and ALP can be affected by factors other than the Zn status of the individual.

Normal serum Zn concentrations are considered to be 0.8 to 1.0 mg/L for sheep (Underwood, 1977). Serum Zn concentrations at the beginning of the study ranged from 0.76 to 0.94 mg/L indicating the lambs were Zn adequate (table 3). Lambs in all the dietary treatment groups maintained normal serum Zn concentrations throughout the study. The results also suggests that the Zn concentration of the basal diet was sufficient to maintain Zn adequacy. As with growth, supplementation of Zn as an organic source resulted in no improvements in serum Zn over that from the inorganic source. Steers fed a corn-cottonseed hulls-cottonseed meal diet (27.8 mg of Zn/kg) had decreased plasma Zn concentrations when compared to steers fed the basal diet supplemented with Zn (50 mg of Zn/kg) either as ZnO or ZnMet (Chirase et al., 1994). As in the present study, plasma Zn concentrations were similar in steers fed the two Zn sources. Zinc methionine also has sustained plasma Zn concentrations in infectious bovine rhinotracheitis virus-stressed steers (Chirase et al., 1994).

Serum cortisol concentrations were unaffected ($p > .05$) by the level and source of Zn supplementation (20.0 ± 3.0, 17.2 ± 3.0 and 21.5 ± 3.0 ng/mL, for basal, ZnO and ZnMet, respectively). However, cortisol concentrations were greater ($p < .05$) 5 h after ACTH administration and initiation of fasting than at 0 or 48 h (30.5 ± 2.9 versus 12.9 ± 2.0 and 15.6 ± 2.0 ng/mL, for 5, 0 and 48 h, respectively). This increase in cortisol concentrations 5 h after administration of ACTH was lower than the 50 to 100 ng/mL observed in experiments with lambs in which lymphocyte function was reduced by restraint and isolation stress (Minton et al., 1995). Infusion of stress-matched concentrations of cortisol had no effect on lymphocyte blastogenesis (Minton et al., 1995). Cortisol concentrations in the present study had returned to almost initial levels by 48 h. This rapid increase and decrease in

serum cortisol indicated the regime utilized (ACTH administration and fasting) may have induced stress in the lambs; however, the duration of the stress was relatively short. Lambs exhibited signs of hunger (i.e., banging feeders and vocalizing in response to the presence of the caretaker); however, no obvious signs of distress were observed. A more prolonged period of stress, with the same or different stressors, may have affected the lambs differently than in the present study.

Table 3. Effect of level and source of zinc supplementation on serum alkaline phosphatase activity and zinc concentrations

Item	Treatment			SEM
	Control	ZnO	ZnMet	
Alkaline phosphatase (U/L)				
d 0	207.6	173.8	175.4	46.1
d 28	241.2	207.1	205.4	16.7
d 57	196.8	195.9	220.5	20.7
d 84	175.4	161.6	209.1	21.7
d 112	158.4	160.7	203.6	18.8
Serum zinc (mg/L)				
d 0	0.94	0.84	0.76	0.09
d 28	0.91	0.94	0.89	0.07
d 57	0.96	0.93	0.85	0.05
d 84	0.89	0.86	0.84	0.05
d 112	0.86	0.87	0.87	0.03

Table 4. Effect of level and source of zinc supplementation on serum zinc concentrations after ACTH administration and fasting[a]

Time	Treatment			SEM
	Control	ZnO	ZnMet	
	Serum zinc			
	..... mg/L			
0 h[b]	0.86	0.87	0.87	0.03
5 h[d]	1.04	1.05	1.03	0.03
48 h[b]	0.94	0.92	0.85	0.03
7 d[c,d]	1.01	1.01	0.98	0.03
14 d[c]	1.00	0.96	0.99	0.03

[a] Blood samples were collected at 0 h on d 112, and the lambs were then administered 100 I.U. of ACTH intramuscularly and fasted for 48 h.

[b,c,d] Times lacking a common superscript letter differ ($p < .01$).

Serum Zn concentrations in response to ACTH administration and fasting for 48 h are shown in table 4. Serum Zn concentrations were unaffected ($p > .05$) by the level and source of dietary Zn supplementation at any sampling time. However, serum Zn concentrations were affected ($p < .01$) by ACTH administration and fasting. At 0 h (administration of ACTH and initiation of fasting) and 48 h post-ACTH and fasting, serum Zn concentrations were at the lowest ($p < .01$) level, whereas 5 h post-ACTH resulted in the greatest ($p < .01$) values. Serum Zn concentrations 7 and 14 d after ACTH administration were greater ($p < .01$) than at 0 and 48 h; however, only values on d 14 were less ($p < .01$) than values at 5 h (0.87 $\pm$ 0.04, 1.04 $\pm$ 0.04, 0.91 $\pm$ 0.03, 1.00 $\pm$ 0.04 and 0.98 $\pm$ 0.04 mg/L, for 0, 5 and 48 h, 7 and 14 d, respectively). A dietary treatment by time interaction was not observed ($p > .05$). Previous research showed that serum Zn initially increased in reponse to dexamethasone, but was not different from initial levels 48 h after administration (Droke et al., 1993). The same finding was observed in the present study with ACTH. Stress (cortisol) and cytokines (interleukin-1) can result in a redistribution of Zn from the plasma to the liver (Pekarek and Evans, 1976; Chesters and Will, 1981; Hempe et al., 1991). The amount of ACTH used in the present study was based on preliminary research (unpublished data) and was known to increase serum cortisol. The period of high cortisol concentrations in the present study may not have been of sufficient duration to induce the redistribution of Zn.

The *in vitro* response of peripheral blood lymphocytes to mitogens ([^{3}H]thymidine incorporation, blastogenesis) was unaffected ($p > .05$) by the level and source of dietary Zn supplementation, either before or after the period of stress (table 5); therefore, the values are presented as the pooled means of pre- and post-ACTH fasting data. Previous research indicated that lambs with clinical signs of Zn deficiency had suppressed lymphocyte blastogenesis in response to T-lymphocyte mitogens (Droke and Spears, 1993). However, later work found blastogenesis to be unaffected by dietary Zn before and after dexamethasone administration in lambs without clinical signs of Zn deficiency (Droke et al., 1993). These results indicate that Zn supplementation of practical diets during periods of stress may not enhance the ability of the sheep lymphocytes to respond to stimulation.

The use of AS instead of FCS resulted in greater ($p = .07$) PWM-induced blastogenesis (table 6). In unstimulated cultures, blastogenesis was greater ($p < .05$) with AS than FCS, before and after ACTH and fasting. In LPS-stimulated cultures, the use of AS resulted in less ($p < .05$) blastogenesis than when FCS was used; however, ACTH and fasting only decreased ($p < .05$) LPS-stimulated blastogenosis when FCS was used. The use of

Table 5. Effect of level and source of zinc on *in vitro* mitogen-induced lymphocyte blastogenesis[a]

Mitogen[b]	Treatment			SEM
	Control	ZnO	ZnMet	
	..... [^{3}/H] thymidine incorporation (cpm)			
Unstimulated	6.35(572)	6.41(602)	6.40(608)	0.08
PHA	10.96(57,526)	10.79(48,533)	10.68(43,478)	0.11
PWM	10.46(34,892)	10.46(34,892)	10.58(39,340)	0.10
LPS	7.57(1,939)	7.70(2,208)	7.70(2,208)	0.16

[a] Values are the ln transformed (back transformed means) means of pooled pre-and post-ACTH fasting and serum source data.
[b] Mitogens used: unstimulated, no mitogen; PHA = phytohemagglutinin; PWM = pokeweed mitogen; LPS = lipopolysaccharide.

Table 6. Effect of ACTH and fasting, and source of assay serum on lymphocyte blastogenesis[a]

Serum[b]	Day[c]	Unstim	PHA	PWM	LPS
		..... [^{3}H] thymidine incorporation (cpm)			
AS	112	5.90(365)[e]	11.04(62,318)	10.34(30,946)	6.65(773)[f]
	114	6.45(633)[d]	10.63(41,357)	10.86(52,052)	6.96(1,054)[f]
FCS	112	6.65(773)[d]	10.95(56,954)	10.09(24,101)	8.85(6,974)[d]
	114	6.56(706)[d]	10.62(40,946)	10.73(45,707)	8.17(3,533)[e]
	SEM	0.11	0.15	0.14	0.21
ANOVA (P-value)					
	Serum	.0001	NS	.0687	.0001
	Day	.0058	.0015	.0001	NS
	Day × Serum	.0002	NS	NS	.0019

[a] Values are the ln transformed means (back transformed means) of pooled dietary treatment data.
[b] AS = autologous serum; FCS = fetal calf serum.
[c] Day 112 = lambs were injected with 100 IU of ACTH intramuscularly and fasted for 48 h.
[d,e,f] Means within a column lacking a common superscript letter are different ($p < .05$).

AS more accurately simulates the *in vivo* conditions the lymphocytes developed in. The differences between the AS and FCS may be due to differences in ligands or the presence or absence of inhibitors. These differences indicate that the use of autologous serum may be more appropriate when trying to determine the effect of a micronutrient on lymphocyte function.

Lumphocyte blastogenesis after ACTH administration and a 48-h fast (d 114) was less ($p < .01$) for PHA- and greater ($p < .01$) for PWM-treated lymphocytes than for lymphocytes collected pre-ACTH and fasting (d 112; table 6). Dexamethasone administration has also been shown to suppress PHA-induced blastogenesis; PWM-induced blastogenesis was unaffected (Droke et al., 1993). These data, along with previous research (Droke et al., 1993; Coppinger et al., 1991) indicate T-lymphocytes may be more susceptible to cortisol-induced immunosuppression than B-lymphocytes.

The humoral immune response as measured by the production of antibodies to porcine RBCs was unaffected ($p > .05$) by the level and source of dietary zinc (table 7). This is in agreement with previous results obtained in lambs marginally deficient in Zn (Droke et al., 1993), but in contrast to an enhanced response in Zn deficient chicks (Pimentel et al., 1991) or a suppressed response in Zn deficient mice (DePasquale-Jardieu and Fraker, 1979). Similar responses among dietary treatments in the present study may be due to the lack of a severe Zn deficiency.

The results of the present study indicate that a practical diet containing approximately 28 mg Zn/kg of diet was adequate to support growth in the lambs. This level of dietary Zn also was sufficient to maintain Zn status and immune function during a period of physiologic stress. No differences in responses were obtained when Zn was supplemented as ZnMet or ZnO. This suggests that even though ZnMet appears to be

Table 7. Effect of level and source of zinc on porcine red blood cell hemagglutination titers[a]

Day	Titer	Treatment			SEM
		Control	ZnO	ZnMet	
		..... titers, log_2			
0	Total	1.0	1.0	1.2	0.1
	IgG	1.0	1.0	1.0	0.0
	IgM	0.0	0.0	0.2	0.1
7	Total	5.4	5.4	5.2	0.4
	IgG	5.2	4.8	4.6	0.6
	IgM	0.4	0.6	0.6	0.3
14	Total	5.2	4.8	4.8	0.5
	IgG	4.4	4.0	3.8	0.6
	IgM	0.8	0.8	1.0	0.2

[a] Day 114-48 h after ACTH administration and fasting, lambs were injected intramuscularly with porcine red blood cells.

metabolized differently than ZnO, supplementation with ZnMet does not appear to affect immune responses of stressed lambs differently than when they are supplemented with ZnO.

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