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Summary

Twelve lactating Holstein cows (132 ± 21 days in milk) were enrolled in a Latin square experiment to explore the extent to which source and amount of supplemental dietary Zn can impact barrier function of mammary epithelial tissue. Cows received either 970 mg supplemental Zn/day as $ZnSO_4$ (LS), 1,640 mg supplemental Zn/day as $ZnSO_4$ (HS), or 1,680 mg supplemental Zn/day as a mixture of $ZnSO_4$ and Zn methionine complex (HC). Treatments lasted for 17 days followed by 4 days of sample collection. Blood and milk were collected and analyzed for markers of blood-milk leak including plasma lactose and α-lactalbumin and milk electrolytes. Total RNA was also isolated from milk cells and abundance of Zn transporter 2 (ZnT2) and clusterin, genes with potential impact on Zn-dependent apoptosis and cell survival, were measured. Finally, dairy food properties of milk (heat coagulation time, nonprotein nitrogen, and non-casein nitrogen) were also analyzed. Cows on the HS treatment tended to have higher feed intake than LS ($P = 0.06$), and milk fat percentage tended to increase for HC compared to LS ($P = 0.08$). No other effects on milk composition, yield, or production efficiency were observed. No effects were observed on markers of blood-milk leak, mRNA abundance of ZnT2 or clusterin, or dairy food chemistry properties. Concentration and source of dietary Zn did not impact mammary epithelial integrity in lactating cows during late lactation.

Key words: zinc, mammary epithelia, apoptosis

Introduction

Dietary Zn is important for immunity, reproduction, hormone activity, and the activity of many enzymes in dairy cattle. Dietary Zn availability can also impact epithelial tissues, such as mammary epithelial cells (MECs), the cells responsible for milk secretion. In basic research conducted on mice, moderate Zn deficiency impacts milk secretion, gross morphology, and apoptosis rate in the mammary gland. Research in dairy cattle nutrition has shown that feeding diets marginally deficient in Zn during early lactation can compromise mammary health. Further, health and production benefits have been shown when feeding amino acid-chelated Zn as opposed to inorganic Zn sources such

[1] Zinpro Corp., Eden Prairie, MN.

as zinc sulfate or zinc oxide. In this study, we explored the extent to which these effects could be mediated by impacts on MEC structure and apoptosis, evaluated in response to feeding two levels of supplemental Zn with varying proportions provided as a Zn methionine complex. We hypothesized that cows fed higher levels or more bioavailable Zn would show fewer signs of epithelial barrier disruption in the mammary gland.

Experimental Procedures

Twelve multiparous Holstein cows in mid- to late-lactation (132 ± 21 days in milk) were enrolled in a three-period Latin square experiment. Throughout the course of the study, cows were housed in individual tie stalls equipped with automatic waterers, fed a balanced basal ration twice daily (Table 1), and milked 3 times daily. Treatments consisted of a low inorganic zinc supplement (970 mg supplemental Zn/day provided as $ZnSO_4$, LS), a high inorganic zinc supplement (1,640 mg Zn/day provided as $ZnSO_4$, HS), and a high Zn supplement partially provided in the form of an amino acid complex (1,680 mg supplemental Zn/day provided as 33% $ZnSO_4$ and 67% Zn-methionine [Zinpro, Zinpro Corp, Eden Prairie, MN] on a Zn basis, HC). Treatments were administered once daily as a gel capsule containing all supplemental trace minerals except for selenium, which was included in the grain mix (Table 2). Each cow received all three treatments and treatments were balanced for potential carryover effects across periods. Each period lasted 21 days, with 17 days of diet adaptation and 4 days of sample collection.

Milk yield, milk composition, and feed intake were recorded and averaged for each cow over each 4-d sampling period. To assess blood-milk leak, sodium and potassium concentrations were measured in milk collected during sampling periods, and blood concentrations of these electrolytes were also measured as covariates for the milk electrolyte statistical analysis. Blood plasma lactose and alpha-lactalbumin were measured as additional markers of milk-blood leak. Heat coagulation time, non-protein nitrogen, and non-casein nitrogen were measured in milk samples to assess dairy food properties of milk. Finally, mRNA abundance of ZnT2 and clusterin, genes related to Zn-mediated apoptosis and cell survival, were measured in cells collected from milk samples during sampling periods.

Data were analyzed in JMP v10.0 (SAS Institute, Cary, NC) with a mixed model using fixed effects of treatment and period, and the random effect of cow. Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$. Interactions of treatment and period were also tested and removed from the model if they did not contribute significantly ($P > 0.10$).

Results and Discussion

Feed intake tended to increase for HS cows ($P = 0.06$) compared to LS, and milk fat percentage tended to increase for HC ($P = 0.08$) compared to LS. No other effects on milk composition, yield, or production efficiency were observed (Table 3). Plasma electrolyte, lactose and α-lactalbumin concentrations were also unaffected by treatment, as were milk electrolytes (Table 4). No treatment effects were observed in transcript abundance of ZnT2 or clusterin in milk cells (Table 4). Finally, no effects of treatments

were observed on heat coagulation time or the proportion of nonprotein nitrogen or noncasein nitrogen in the milk (Table 5).

Results from this study indicate that supplemental zinc source and level in practical diets of mid-lactation dairy cattle have little effect on the integrity of the blood-milk barrier of the healthy mammary gland. In previous research, plasma lactose and α-lactalbumin levels have been shown to increase with disruption of the epithelial barrier separating blood from milk in the mammary gland. The lack of treatment effects observed in this study likely indicates that epithelial integrity was not appreciably affected by our treatments, perhaps in part due to the use of healthy, non-mastitic cows. In the present study, all cattle were fed a diet which met predicted requirements for zinc intake, and treatment diets were administered for only a portion of the lactation period, in order to facilitate a Latin square experimental design.

Feeding of marginally zinc-deficient diets in early lactation has been shown to increase somatic cell counts in dairy cattle. Further, multiparous cows supplemented with an increased proportion of zinc as zinc methionine have reduced somatic cell counts and higher colostrum immunoglobulin G, indicating improved mammary health and possibly enhanced immune function. One possible explanation for these observations is that zinc supplementation leads to less apoptosis in the mammary gland, leading to a tighter blood-milk barrier. Marginally zinc deficient mice have been shown to experience dramatic increases in apoptosis rates in mammary tissue leading to differences in gross morphology of the mammary gland during lactation. If this were the case for our model, we would likely have observed differences in markers of blood-milk leak.

Another potential explanation for zinc supplementation leading to improved mammary health is a direct impact on immune responses. Dietary zinc levels impact lymphocyte numbers as well as other aspects of the immune response in mice; however, zinc supplementation in practical diets of cattle does not always impact measures of immune function. It is possible that improvements in mammary health and milk production that sometimes come with zinc supplementation are the product of improved immune response to mastitis challenge, without directly affecting apoptosis of mammary epithelial cells or integrity of the blood-milk barrier.

Transcript abundance of selected genes was determined as a further exploratory attempt to discover cellular pathways impacted by zinc supplementation in the mammary gland. Collecting RNA from an internal tissue of a living organism is typically a difficult task, requiring a biopsy procedure. In the case of mammary epithelial cells (MEC), there are alternatives to biopsy, which in turn present their own challenges. In the present study, we utilized milk somatic cells as a source of mammary tissue. Isolation of RNA from milk somatic cells has been shown to produce RNA sequencing results comparable to other more intensive sampling methods such as biopsies, laser-capture of cells from fixed tissue, and antibody-capture of milk epithelial cells. However, in a healthy cow, less than 10% of milk somatic cells are expected to be MEC, with the balance largely made up of immune cells. It is possible that transcript abundance of genes expressed preferentially in MEC could be diluted by the low proportion of MEC in milk somatic cells. Abundance was low for both ZnT2 and clusterin, which may partially explain why no differences were detected.

Conclusion

Zinc supplementation of dairy rations at 950 mg/day as opposed to 1,650 mg/day did not appear to impact the integrity of the blood-milk barrier or dairy food properties of milk, nor did the inclusion of a greater proportion of the Zn as an amino acid complex. Milk production responses to amino acid-bound Zn sources which have been documented in studies with larger sample sizes are likely due to mechanisms other than a direct impact on mammary epithelial integrity. However, it is possible that these treatments could elicit a response in cohorts with greater mastitis pressure, with more challenges to mammary epithelial integrity.

Table 1. Formulation and composition of basal diet

Item	Value
Ingredient, % of diet DM	
Alfalfa hay	18.64
Corn silage	18.85
Wet corn gluten feed[1]	24.29
Cotton seed	4.28
Lactation grain mix[2]	33.94
DM, %	53.37
Nutrient, % of diet DM	
Crude protein	17.94
Acid detergent fiber	19.33
Neutral detergent fiber	30.40
Lignin (sulfuric acid)	4.70
Non-fiber carbohydrate	42.13
Fat (ether extract)	4.43
Zinc, ppm	58.33
Net energy for lactation,[3] Mcal/lb	0.72

[1]Sweet Bran (Cargill Inc., Blair, NE).
[2]Lactation grain mix consisted of 66.2% fine rolled corn, 20.2% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 4.04% limestone, 0.5% stock salt, 0.5% potassium chloride, 3.53% sodium bicarbonate, 0.81% magnesium oxide, 0.13% selenium premix (0.06%), 0.05% vitamin A premix (30 kIU/g), 0.02% vitamin D premix (30 kIU/g), 0.5% vitamin E premix (20 kIU/g), 0.02% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.63% XP Yeast (Diamond V, Cedar Rapids, IA), 0.32% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), and 2.52% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ).
[3]Estimated according to NRC (2001).

Table 2. Composition of treatment capsules

Item[1]	30-ZS	60-ZS	60-ZC
% DM	97.97	98.28	97.74
Ca, % DM	1.55	1.33	5.23
P, % DM	0.01	0.01	0.01
Mg, % DM	0.13	0.10	0.11
K, % DM	0.23	0.21	0.26
S, % DM	15.42	15.31	10.35
Mn, % DM	12.84	9.95	9.69
Zn, % DM	7.99	11.55	10.37
Cu, % DM	1.89	1.40	1.32
Fe, % DM	0.23	0.26	0.23
Na, % DM	2.61	1.94	2.01
Cl, % DM	2.32	2.42	8.83
DCAD, mEq/100 g	909.72	935.44	801.66
Dose, grams/day			
Ca	0.19	0.19	0.85
P	0.00	0.00	0.00
Mg	0.02	0.01	0.02
K	0.03	0.03	0.04
S	1.87	2.17	1.68
Mn	1.56	1.41	1.57
Zn	0.97	1.64	1.68
Cu	0.23	0.20	0.21
Fe	0.03	0.04	0.04
Na	0.32	0.28	0.32
Cl	0.28	0.34	1.43
Met	2.71	2.71	2.69
Metabolizable Met	2.19	2.19	2.18

[1] Values are results of analysis of mineral mixes (Dairyland Laboratories, Arcadia, WI).

Table 3. Intake and milk production

	Least square means				*P* - value	
Item	30-ZS	60-ZS	60-ZM	SEM	Trt	Period
DMI, lb/day	62.1	64.0	63.7	1.4	0.06	< 0.001
Milk, lb/day	104.1	104.4	105.4	3.0	0.74	0.16
ECM, lb/day	105.9	107.8	109.3	2.6	0.36	0.23
Milk/DMI	1.68	1.63	1.66	0.04	0.13	< 0.001
ECM/DMI	1.71	1.69	1.73	0.03	0.42	< 0.001
Protein, %	3.04	3.03	3.02	0.06	0.52	< 0.001
Protein, lb/day	3.15	3.15	3.18	0.09	0.97	0.25
Fat, %	3.56	3.69	3.76	0.13	0.08	0.35
Fat, lb/day	3.68	3.84	3.92	0.13	0.15	0.17
Lactose, %	4.90	4.94	4.91	0.04	0.21	0.098
Lactose, lb/day	5.09	5.14	5.16	0.13	0.71	0.09
MUN, mg/dL	13.15	13.53	13.49	0.50	0.23	0.21

Table 4. Markers of mammary epithelial integrity

	Least square means				*P* - value	
Item	30-ZS	60-ZS	60-ZM	SEM	Trt	Period
Blood K, mM	4.35	4.59	4.36	0.09	0.12	0.11
Blood Na, mM	136.86	136.47	136.31	0.38	0.52	0.57
Blood Cl[3], mM	99.52	99.84	99.82	0.52	0.80	0.43
Blood Hb,[3] *mM	5.62	5.70	5.67	0.07	0.48	0.04
Blood Hct[3] (% PCU)	26.58	27.00	26.85	0.33	0.48	0.04
Plasma lactose, μM	21.32	20.91	19.14	2.35	0.73	0.53
Milk Na,[1] ppm	354	349	369	11	0.15	< 0.001
Milk K, ppm	1516	1492	1527	30	0.55	< 0.01
ZnT2, NTA[2]	0.59	0.39	0.35	0.15	0.25	0.054
Clusterin, NTA	2.27	0.98	1.20	0.72	0.41	< 0.01

[1]Modeled with blood Na as a covariate ($P < 0.01$).
[2]NTA = Normalized transcript abundance.
[3]Treatment × period effect significant and included in the model ($P < 0.1$).