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Article *in* The Veterinary Journal · May 2014

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The effect of injectable trace minerals (selenium, copper, zinc, and manganese) on peripheral blood leukocyte activity and serum superoxide dismutase activity of lactating Holstein cows

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ARTICLE INFO

Article history:
Accepted 14 February 2014

Keywords:
Trace minerals
Neutrophil activity
Superoxide dismutase
Oxidative stress
Mastitis

ABSTRACT

The objective of this study was to evaluate the effect of subcutaneous supplementation of 300 mg of zinc, 50 mg of manganese, 25 mg of selenium, and 75 mg of copper on peripheral blood leukocyte activity and serum β-hydroxybutyrate (BHBA) concentrations at 10 ± 2 days in milk (DIM), and on serum superoxide dismutase (SOD) activity during the transition period and subsequent lactation of multiparous Holstein cows. A total of 250 multiparous cows were randomly allocated into one of two treatments groups, namely, trace mineral supplemented (TMS) or control. Cows in the TMS group were injected at 230 and 260 days of gestation, and 35 days postpartum. Serum SOD activity was measured at enrollment, and 10, 60 and 100 DIM. Serum BHBA concentration and leukocyte function were assessed at 10 DIM. Overall serum SOD activity for TMS and control was 16.01 and 12.71 U/mL, respectively. The interaction between treatment and time of serum collection was significant. Additionally, overall serum SOD activity was 12.85 and 14.78 U/mL for cows diagnosed with mastitis and unaffected cows, respectively. Treatment did not affect leukocyte function. For parity >2, TMS cows had lower serum BHBA concentrations than control cows; BHBA concentrations were 0.41 and 0.27 mmol/L for control and TMS cows, respectively. In conclusion, cows diagnosed with mastitis had decreased serum SOD activity, and trace mineral supplementation increased serum SOD activity although leukocyte function was not affected by supplementation.

Introduction

The transition period (defined as the period from 3 weeks before to 3 weeks after calving) is extremely challenging for the dairy cow (Drackley, 1999). It has been reported that the concentration of some trace minerals are affected during the transition period, especially around the time of parturition (Goff and Stabel, 1990; Xin et al., 1993; Meglia et al., 2001).Trace minerals play an important role in dairy cow immune function (Shankar and Prasad, 1998), fertility (Rabiee et al., 2010), and growth (Enjalbert et al., 2006). Polymorphonuclear leukocyte function and bactericidal efficiency is compromised during the transition period (Burvenich et al., 2003). Trace mineral status, especially selenium (Se) and zinc (Zn), affects neutrophil function in postpartum cows and affects neutrophil adhesion and superoxide production (Meglia et al., 2001; Cebra et al., 2003).

Recently, we conducted a large field trial using 1400 dairy cows from three farms located in upstate New York to evaluate the effect of a trace mineral product containing Zn, Se, manganese (Mn) and copper (Cu) at 230 and 260 days of gestation, and 35 days postpartum on production, fertility and health traits. Supplementation did not affect milk production or fertility; however, we observed a significant positive effect on udder health for multiparous cows, decreasing somatic cell count (SCC) and the incidence of clinical mastitis (Machado et al., 2013). Nevertheless, the effect of injectable trace mineral on the transition cow's immune and antioxidant systems remains unknown.

Metabolic demands associated with the transition period increase the production of reactive oxygen species (Sordillo et al., 2009) and may lead to oxidative stress (Miller et al., 1993). Superoxide dismutases (SOD) are enzymes that are involved in the antioxidant system and are Mn, Cu and Zn dependent (Andrieu, 2008). The enzymes are considered to be the first line of antioxidant defense, converting superoxide anion (O_2^-) to hydrogen peroxidase (H_2O_2), which is not a free radical (Michiels et al., 1994). Although there is evidence for a role of other antioxidants in udder health,

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http://dx.doi.org/10.1016/j.tvjl.2014.02.026
1090-0233/

Table 1
Chemical composition (mineral and vitamins) of pre-fresh and fresh cow diets.

Chemical composition (dry matter basis)	Pre-fresh	Lactation
Crude protein (%)	14.00	15.70
Soluble protein (% CP)	30.00	29.00
Acid detergent fiber (%)	26.40	20.50
Neutral detergent fiber (%)	43.00	33.00
Calcium (%)	1.35	0.86
Phosphorus (%)	0.40	0.43
Magnesium (%)	0.35	0.31
Potassium (%)	1.19	1.35
Sodium (%)	0.18	0.49
Sulfur (%)	0.43	0.27
Copper (ppm)	15.00	20.00
Iron (ppm)	292.00	188.00
Manganese (ppm)	85.00	63.00
Selenium (ppm)	0.66	0.80
Zinc (ppm)	74.00	103.00
Molybdenum (ppm)	0.90	1.20

Pre-fresh diets were fed from 3 week prepartum through parturition, and lactation diets were fed from parturition through week 35 postpartum.

the effect of SOD status on mammary gland health deserves further research (Sordillo and Aitken, 2009).

A high serum concentration of postpartum β-hydroxybutyrate (BHBA) is an indicator of negative energy balance in the transition period, and it is associated with increased fat mobilization in early lactation (Ingvartsen and Andersen, 2000). It has been previously reported that Cu supplementation increased metabolism of adipose tissue in steers, due to an increased response to hormones responsible for lipolysis (Engle et al., 2000). To the best of our knowledge, there is no information regarding what effect Mn, Zn, Se, and Cu supplementation during the transition period has on postpartum BHBA concentrations.

The objective of this study was to evaluate the effect of subcutaneous supplementation of 300 mg of Zn, 50 mg of Mn, 25 mg of Se, and 75 mg of Cu (Multimin 90, Multimin North America) at 230 and 260 days of gestation, and 35 days postpartum on peripheral blood leukocyte activity and serum BHBA concentration at 10 ± 2 days in milk (DIM), and serum SOD activity during the transition period, and subsequent lactation of multiparous Holstein cows.

Material and methods

Farms and management

This study was conducted at a dairy farm located near Ithaca, New York. Cows were enrolled from 24 August until 29 September 2011; the follow-up period continued until 4 July 2012. The farm milked 3300 Holstein cows three times daily in a double 52-stall parallel milking parlor. The cows were housed in freestall barns, with concrete stalls covered with mattresses and bedded with manure solids. All cows were offered a total mixed ration (TMR) consisting of approximately 55% forage (corn silage, haylage and wheat straw) and 45% concentrate (corn meal, soybean meal, canola, cottonseed and citrus pulp) on a dry matter basis. The diet was formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% fat corrected milk. Nutrient contents of the diets are described in Table 1.

Samples of TMR from pre-fresh and lactation diets were obtained on two different days of a given week during the study period. The samples were combined into 2-day composite samples and submitted (for both pre-fresh and lactation diets) to a commercial laboratory (Dairy One Cooperative) for wet chemistry analysis. Samples were analyzed for dry matter, crude protein, acid detergent fiber, neutral detergent fiber, and macro and micro minerals.

Study design and treatments

A randomized field trial study design was used; 250 cows were randomly allocated by study entry date into one of two treatments groups: trace mineral supplemented (TMS) or control. Randomization was completed in Excel (Microsoft) and imported into the farm's Dairy Comp 305 program (Valley Agricultural Software). Cows that were randomly assigned to the treatment group received three injections of 5 mL of Multimin 90 at 230 and 260 days of gestation, and 35 days postpartum; each injection contained 300 mg Zn, 50 mg Mn, 25 mg Se, and 75 mg Cu.

Body condition scores (BCS) were determined for all study cows at 230 days of gestation and at 35 ± 3 DIM by a single investigator masked to the treatment group using a five-point scale with a quarter-point system (Edmonson et al., 1989). Serum samples were collected at 230 ± 3 and 260 ± 3 days of gestation, 10 ± 2, 60 ± 3, and 100 ± 3 DIM. For evaluation of peripheral blood neutrophil function, blood was collected at 10 ± 2 DIM and processed for leukocyte activity within 15 h.

The proposal was approved by the Cornell University Animal Care and Use Committee (2009-0001) and owner consent was obtained before the study was started.

Case definition

Retained fetal membranes, metritis, ketosis, displaced abomasum and clinical mastitis were diagnosed and treated by trained farm personnel who followed a specific diagnostic protocol designed by veterinarians from the Ambulatory and Production Medicine Clinic, Cornell University. Farm personnel were masked to the treatments. The presence of retained fetal membranes was defined as failure to release fetal membranes within 24 h of calving. Metritis was defined as the presence of fetid, watery, red-brown uterine discharge and rectal temperature >39 °C. Ketosis was defined as high concentrations of ketone bodies (≥1470 μmol/L) in urine using Ketostix (Bayer). Displaced abomasum diagnosis made by the farm personnel was confirmed by veterinarians. Clinical mastitis was defined as abnormal changes in the udder and milk, such as watery appearance, flakes and clots. Data regarding health traits during the follow-up period were extracted from the farm's DairyComp 305 database, and all health events were considered as a single event variable.

Peripheral blood leukocyte function, serum SOD activity and serum concentration of BHBA

Leukocyte phagocytic activity was evaluated at 10 DIM using Phagotest Kit (Orpegen Pharma) containing fluorescein-labeled opsonized *Escherichia coli* (*E. coli* – FITC), following the manufacturer's instructions. Granulocyte oxidative burst was determined quantitatively with Bursttest Kit (Orpegen Pharma) following the manufacturer's instructions. Cells were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using a 488 nm argon-ion laser. Ten thousand events were collected for each cell population (neutrophils or monocytes). Neutrophil activity assays were only performed on the first 200 cows that entered the study; one cow was excluded from these analyses due to a laboratory error.

Serum SOD activity was assessed using Superoxide Dismutase Assay Kit (Cayman Chemical Company), following the manufacturer's instructions. Serum SOD activity was measured at 230 ± 3 days of gestation, and 10 ± 2, 60 ± 3, and 100 ± 3 DIM.

Serum concentration of BHBA was measured using the Auto kit 3-HB (Wako Chemicals), following the manufacturer's instructions. Serum concentration of BHBA was measured at 10 ± 2 DIM. Serum concentrations of BHBA were only performed on the first 200 cows that entered the study.

Statistical analyses

To facilitate data analysis and interpretation, the variables BCS at enrollment (BCS1 = 1 if BCS was < 3; BCS1 = 2 if BCS = 3; BCS1 = 3 if BCS > 3), BCS at 10 ± 2 DIM (BCS2 = 1 if BCS at 10 ± 2 DIM was less than 3; BCS2 = 2 if BCS = 3 BCS2 = 3 if BCS > 3), and Disease (Disease = 0 if the cow did not have any health event during the follow up period; Disease = 1 if the cows had at least one health event) were created. Descriptive statistics analysis was undertaken in SAS using the FREQ procedure (SAS Institute). The statistician was not blinded to the treatments. The experimental unit was the cow.

Two mixed general linear models were fitted to the data using the MIXED procedure of SAS. The dependent variable evaluated in these analyses was serum SOD activity. The model assumption of normally distributed residuals was satisfied by visual evaluation of the distribution plot of the studentized residuals. The data were longitudinally collected and comprised a series of repeated measures of the dependent variable throughout the four time points of serum collection: at 230 days of pregnancy (enrollment), and 10 ± 2, 60 ± 3, and 100 ± 3 DIM. To account appropriately for repeated measures, the error term was modeled by imposing a first-order autoregressive covariance structure for all statistical models.

The independent variables offered to the first model were: treatment, BCS1, parity, and time of serum collection. The independent variables offered to the second model were: BCS1, BCS2, parity, ketosis, mastitis, metritis, retained placenta, displaced abomasum and time of serum collection. Two-way and three-way interactions between treatment and health parameters, parity and time of serum collection were offered to the models. Furthermore, variables and their respective interaction terms in all models were retained in the model only when they had a significant effect ($P < 0.05$).

To assess the effect of treatment, parity, disease, retained placenta, metritis, mastitis, displaced abomasum and ketosis on the proportion of neutrophils or

monocytes in the blood, percentage of neutrophils or monocytes that performed phagocytosis, mean fluorescence intensity and percentage of neutrophils or monocytes producing reactive oxygen metabolites, and amount of cleaved substrate by neutrophils or monocytes, the ANOVA function of JMP PRO9 (SAS Institute) was used.

A linear regression model was fitted in SAS using the MIXED procedure to assess the effect of treatment on serum BHBA concentration at 10 ± 2 DIM. The independent variables treatment, parity, ketosis, mastitis, metritis, retained placenta, displaced abomasum were offered to the model. Variables were manually and stepwise removed from the model when the *P*-value > 0.05. Two-way interactions between treatment and all other independent variables were added to the model.

Results

Descriptive statistics regarding average age at enrollment (days), average BCS at enrollment and at 10 ± 2, average gestation length at enrollment, number of animals enrolled in parity 2 and >2 are presented in Table 2.

The effect of treatment on serum SOD activity is presented in Fig. 1. Trace mineral supplemented cows had greater overall serum SOD activity than control cows; overall serum SOD activity for TMS and control was 16.01 (SEM = 0.32) and 12.71 (SEM = 0.34) U/mL, respectively ($P < 0.0001$). The interaction between treatment and time of serum collection was significant ($P < 0.0001$).

The effect of parity on serum SOD activity is presented in Fig. 2. Overall serum SOD activity from cows in parity 2 was not different from cows in parity >2; overall serum SOD activity was 14.53 (SEM = 0.30) and 14.20 (SEM = 0.32) U/mL for cows in parity 2 and >2, respectively ($P = 0.44$). However, the interaction between parity and time of serum collection was significant ($P = 0.02$).

The difference in serum SOD activity between cows with and without mastitis is presented in Fig. 3. Overall serum SOD activity was lower for cows diagnosed with mastitis than for cows that did not have mastitis (12.85 (SEM = 0.62) and 14.78 (SEM = 0.26) U/mL, respectively; $P = 0.004$). The interaction between the variable mastitis (yes or no) and the time of serum collection was not significant ($P = 0.33$).

The effects of treatment, parity and health traits on several parameters assessed by the Phagotest assay are presented in Table 3. Treatment did not have any effect on any parameter; however, other traits were associated with some parameters. The effects of treatment, parity and health traits on several parameters assessed by the Bursttest assay are presented in Table 4. Treatment did not have any effect on any parameter ($P \geq 0.05$).

The effect of treatment on serum BHBA concentration at 10 ± 2 DIM is presented in Fig. 4. The interaction between treatment and parity was significant ($P = 0.049$). Control cows had higher mean serum BHBA concentration than TMS cows; however, this difference was significant only for cows in their third or greater parity. Briefly, for cows in parity 2, BHBA concentrations were 0.30 (SEM = 0.02) and 0.25 (SEM = 0.02) mmol/L for control and TMS cows, respectively. For cows with parity > 2, BHBA concentrations were 0.41 (SEM = 0.02) and 0.27 (SEM = 0.02) mmol/L for control and TMS cows, respectively.

Table 2
Descriptive statistics of treatment groups.

	Controls	Trace mineral supplemented
Average age (days) at enrollment (±SE)	1341 (±33)	1332 (±30)
Average body condition score at enrollment (±SE)	3.29 (±0.04)	3.26 (±0.04)
Average body condition score at 10 ± 2 (±SE)	2.90 (±0.04)	2.91 (±0.03)
Average days of gestation at enrollment (±SE)	225.2 (±0.39)	225.5 (±0.35)
Enrolled animals on parity 2 (%)	57 (46)	67 (54)
Enrolled animals on parity > 2 (%)	56 (44)	70 (56)
Total enrolled animals (%)	113 (45)	137 (55)

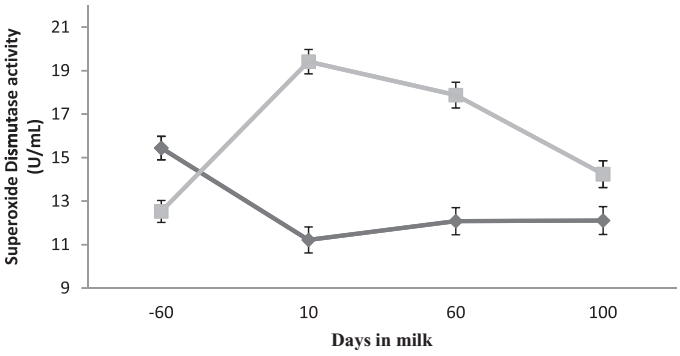

Fig. 1. Effect of treatment on serum superoxide dismutase (SOD) activity (U/mL); the light grey bar illustrates the average SOD activity for cows treated with injectable trace minerals at 60 days before parturition, 30 days before parturition and 35 days in milk, and the dark gray line illustrates the SOD concentration for the control cows. Trace mineral supplemented cows had greater overall serum SOD activity than control cows ($P < 0.0001$). The interaction between treatment and time of serum collection was significant ($P < 0.0001$). Error bar represents the standard error of the means.

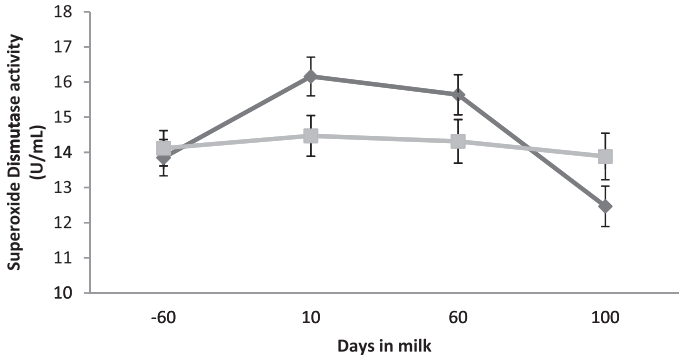

Fig. 2. Effect of parity on serum superoxide dismutase (SOD) activity (U/mL); the light gray bar illustrates the average SOD activity for cows in parity 2 and the dark gray line illustrates the SOD concentration for the cows in parity > 2. Overall serum SOD activity from cows in parity 2 was not different from cows in parity > 2 ($P = 0.44$). However, the interaction between parity and time of serum collection was significant ($P = 0.02$). Error bar represents the standard error of the means.

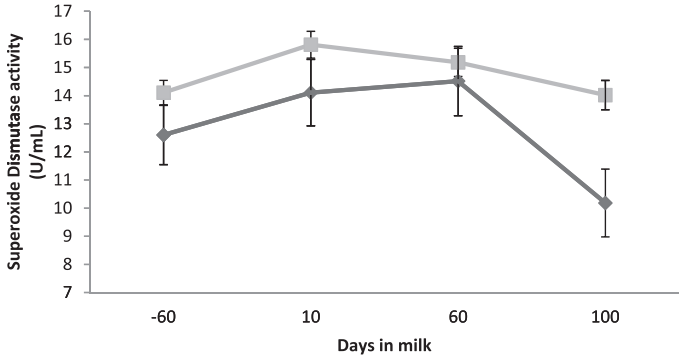

Fig. 3. Serum superoxide dismutase (SOD) activity (U/mL) for cows diagnosed as having or not having mastitis; the light gray bar illustrates the average SOD activity for cows that did not have mastitis and the dark gray line illustrates the SOD concentration for the cows that had mastitis. The overall serum SOD activity was lower for cows diagnosed with mastitis than for cows that did not have mastitis ($P = 0.004$). The interaction between the variable mastitis (yes or no) and time of serum collection was not significant ($P = 0.33$). Error bar represents the standard error of the means.

Table 3
Effect of treatment, parity, ketosis, displaced abomasum, mastitis, metritis and retained placenta on several parameters assessed by the Phagotest assay (phagocytosis). Bold font indicates $P < 0.05$.

		Phagotest					
	Level (*n*)	%N	%M	NP	MP	NM	MM
Treatment	TMS (109)	37.6%	31.7%	83.4%	25.1%	206.4	65.2
	Control (90)	34.2%	32.7%	82.4%	23.8%	201.3	63.5
Parity	2 (106)	**33.9%**	32.2%	83.0%	24.3%	203.5	**61.7**
	>2 (93)	**38.5%**	32.1%	82.8%	24.8%	204.7	**67.6**
Disease	No (136)	36.4%	**30.8%**	83.3%	23.9%	204.4	64.3
	Yes (63)	35.4%	**34.9%**	82.1%	25.7%	203.5	64.8
RFM	No (180)	36.1%	31.8%	83.4%	24.4%	204.9	64.4
	Yes (19)	36.0%	34.9%	78.3%	26.0%	196.0	64.7
Metritis	No (176)	**36.9%**	32.2%	83.2%	24.1%	202.3	63.8
	Yes (23)	**29.9%**	31.8%	81.1%	27.4%	217.7	69.4
Mastitis	No (171)	35.6%	31.5%	83.2%	24.7%	206.8	64.8
	Yes (28)	39.0%	35.7%	81.2%	23.2%	187.8	62.5
DA	No (197)	36.2%	**31.9%**	82.9%	24.5%	204.3	64.4
	Yes (2)	26.7%	**50.7%**	83.3%	22.3%	185.1	67.8
Ketosis	No (185)	**36.7%**	32.0%	82.7%	24.2%	203.4	64.2
	Yes (14)	**28.1%**	33.5%	85.8%	28.3%	213.2	68.0

TMS, trace mineral supplemented. Cows that were randomly assigned to the treatment group received three injections of 5 mL of Multimin 90 (Multimin North America) at 230 and 260 days of gestation, and 35 days postpartum; each injection contained 300 mg Zn, 50 mg Mn, 25 mg Se, and 75 mg Cu; RFM, retained fetal membranes; DA, displaced abomasum; %N, proportion of neutrophils in the blood after removal of erythrocytes; %M, proportion of monocytes in the blood after removal of erythrocytes; NP, percentage of neutrophils having performed phagocytosis; MP, percentage of monocytes having performed phagocytosis; NT, mean fluorescence intensity (reflects number of ingested bacteria) of neutrophils; MM, mean fluorescence intensity (reflects number of ingested bacteria) of monocytes.

Table 4
Effect of treatment, parity, ketosis, displaced abomasum, mastitis, metritis and retained placenta on several parameters assessed by the Bursttest assay (oxidative burst). Bold font indicates $P < 0.05$.

		Bursttest			
	Level (*n*)	OBNP	OBMP	OBNM	OBMM
Treatment	TMS (109)	77.3%	17.9%	25.9	12.3
	Control (90)	78.2%	20.2%	28.6	9.2
Parity	2 (106)	77.5%	19.2%	26.2	11.5
	>2 (93)	78.0%	18.6%	27.9	10.3
Disease	No (163)	78.8%	19.3%	28.1	11.2
	Yes (36)	75.3%	18.1%	24.6	10.4
RFM	No (180)	**78.5%**	18.9%	27.3	11.0
	Yes (19)	**70.7%**	19.6%	23.5	10.2
Metritis	No (176)	78.3%	19.0%	27.5	11.1
	Yes (23)	73.0%	18.3%	23.2	9.8
Mastitis	No (171)	77.4%	19.2%	27.1	10.9
	Yes (28)	79.5%	17.5%	26.3	10.9
DA	No (197)	77.9%	19.1%	27.1	10.9
	Yes (2)	59.6%	6.7%	18.2	7.7
Ketosis	No (185)	78.0%	19.1%	27.3	11.1
	Yes (14)	74.6%	16.4%	22.5	8.0

TMS, trace mineral supplemented. Cows that were randomly assigned to the treatment group received three injections of 5 mL of Multimin 90 (Multimin North America) at 230 and 260 days of gestation, and 35 days postpartum; each injection contained 300 mg Zn, 50 mg Mn, 25 mg Se, and 75 mg Cu; RFM, retained fetal membranes; DA, displaced abomasum; OBNP, percentage of neutrophils having produced reactive oxygen metabolites; OBMP, percentage of monocytes having produced reactive oxygen metabolites; OBNM, mean fluorescence intensity (amount of cleaved substrate, activity) of neutrophils; OBMM, mean fluorescence intensity (amount of cleaved substrate, activity) of monocytes.

Discussion

This study found an association between serum SOD activity and mastitis and an effect of trace mineral supplementation on serum SOD activity. There is evidence for the role of other antioxidants in udder health, such as vitamin C (Weiss and Hogan, 2007), Se and vitamin E (Smith et al., 1984). However, information regarding the association of SOD and udder health is scarce (Sordillo and Aitken, 2009). In the present study, cows that were not affected with mastitis in the subsequent lactation had increased level of serum SOD activity through the four data collection points; however, this difference was significant only at 100 ± 3 DIM. This result suggests that SOD may play a role on udder health, being part of the antioxidant defense system (Sordillo and Aitken, 2009).

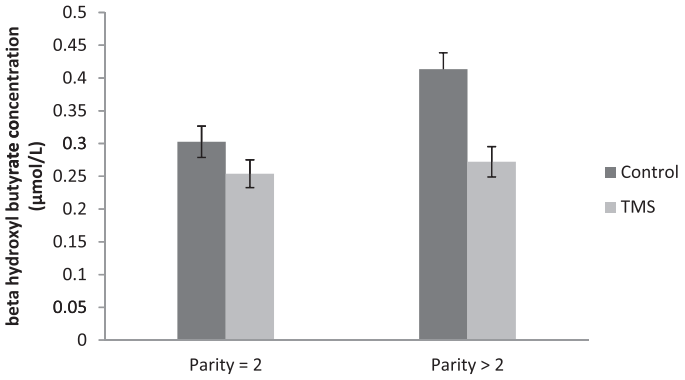

Fig. 4. Effect of treatment on serum β-hydroxybutyrate concentrations at 10 ± 2 days in milk. The interaction term treatment × parity was significant with a larger effect of treatment observed for cows with parity > 2 ($P = 0.049$).

In the present study, TMS cows had increased SOD activity for up to 100 DIM. Prasad and Kundu (1995) reported that weaned calves fed milk supplemented with Zn and Cu had increased blood SOD activity compared with non-supplemented calves. Additionally, ewes had increased plasma SOD activity when they were supplemented with Cu and Zn over the basal diet (Pal et al., 2010). Moreover, Cu supplementation appeared to have a beneficial effect on serum SOD activity for Cashmere goats (Zhang et al., 2012). These studies support our findings, suggesting that at least the supplemented Zn and Cu are being incorporated in the system, resulting in increased SOD activity.

Here we evaluated a multimineral supplement; therefore, it is not possible to relate any response to a particular mineral. However, the study was performed in well managed farms, which did not have any history of mineral deficiencies and where the diets were also well managed. In a recently published study using the same product, it was reported that Se and Cu were increased in the liver over 15 days (Pogge et al., 2012).

Blood Se and Zn concentrations are associated with neutrophil adhesion of periparturient cows (Meglia et al., 2001; Cebra et al., 2003). However, in the present study, trace mineral supplementation did not alter either the phagocytic function or the oxidative burst of leukocytes. Previous studies reported that leukocyte function was increased in cows supplemented with trace minerals (Gyang et al., 1984; Grasso et al., 1990; Hogan et al., 1990) However, these studies reported the effect of oral supplementation (Grasso et al., 1990; Hogan et al., 1990) and the effect of supplementation on trace mineral deficient cows (Gyang et al., 1984; Grasso et al., 1990), while our study evaluated the effect of injected supplementation on dairy cows that were apparently not deficient.

Interestingly, TMS cows tended to have lower serum BHBA concentration than control cows; this difference was significant for cows in their third or greater parity. This result was not expected, because it has been reported that Cu supplementation increases lipolysis in steers (Engle et al., 2000; Stahlhut et al., 2006). It is not clear how trace mineral supplementation decreased BHBA concentration; however, it has been demonstrated that oxidative status is associated with metabolic status in the periparturient period (Bernabucci et al., 2005; Pedernera et al., 2010).

Conclusions

This study showed that cows injected with a trace mineral supplement at 230 and 260 days of gestation, and 35 days postpartum had increased SOD activity through to 100 DIM. Additionally, cows that were affected with mastitis in the subsequent lactation had decreased serum SOD activity. For cows with parity > 2, TMS cows had lower serum BHBA concentrations than control cows. However, leukocyte function was not affected by trace mineral supplementation.

Conflict of interest statement

This study was funded by Multimin North America. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgment

This study was funded by Multimin North America.

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